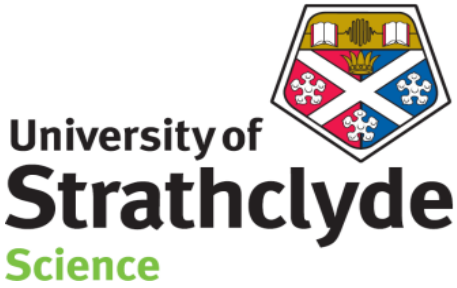

MOLECULAR MECHANISMS OF CELL PROLIFERATION IN ENDOMETRIOSIS

A thesis presented by

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This thesis is the result of author's original research. It has been composed by the author and has not been previously submitted for the examination which has lead to the award of a degree.

Signed:  Date: 25 th August 2011

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ABBREVIATIONS

17β-HSD	17β-hydroxysteroid dehydrogenase
20α-OHP	20α-hydroxyprogesterone
µl	microlitre
µM	micromolar
AA	arachidonic acid
AF-1	activation function-1
AFS	American Fertility Society
AKR	aldoketo reductase
ANOVA	analysis of variance
AP-1	activator protein-1
Apaf-1	apoptosis protease activating factor-1
ARE	antioxidant response element
ASRM	American Society of Reproductive Medicine
ATP	adenosine 5'-triphosphate
Bax	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma 2
BclXL	B-cell leukaemia XL protein
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CBP	cAMP-Response Element-Binding Protein
^{o}C	degree Celsius
CAT	catalase
COX-2	cyclooxygenase-2
CYP	cytochrome 450
Cys	Cysteine
Cyt *c*	cytochrome *c*
DBD	DNA-binding domain
dH_2O	distilled water

DMEM	Dulbecco's Modified Eagle's Medium
DMSO	dimethyl sulfoxide
DTNB	5,5'-dithiobis-2-nitrobenzoic acid
E1	estrone
E2	estradiol
E3	estriol
EDTA	ethylenediaminetetraacetic acid
EIA	Enzyme Immunoassay
Elk1	E twenty-six (ETS)-like transcription factor 1
ER	estrogen receptor
ERE	estrogen response element
ERK	extracellular signal-regulated kinases
ESHRE	The European Society of Human Reproduction and Embryology
FADD	Fas-associating protein with a novel death domain
FBS	fetal bovine serum
Fe^{2+}	Iron (II) ion
Fe^{3+}	Iron (III) ion
FITC	Fluorescence Isothiocyanate
FSH	follicular stimulating hormone
g	gram
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GnRH	gonadotrophin releasing hormone
GPR30	G-protein coupled receptor 30
GRed	glutathione reductase
GSH	reduced glutathione
GSSG	glutathione disulfide (oxidised glutathione)
GST	glutathione S-Transferase
GPx	glutathione peroxidase
H_2O_2	hydrogen peroxide

HO-1	haem oxygenase-1
HSP	heat-shock protein
JNK	c--Jun NH2-terminal kinase
Keap1	Kelch-like ECH associating protein 1
LBD	ligand-binding domain
LDH	lactate dehydrogenase
LH	luteinizing hormone
LPP	lipid peroxidation product
LSB	Laemmli sample buffer
M	molar
MAPK	mitogen activated protein kinase
min	minutes
ml	millilitre
mM	millimolar
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
NAC	N-acetylcysteine
$NADP^+$	Nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	natural killer
nM	nanomolar
Nrf2	nuclear factor erythroid 2-related factor 2
$O_2\bullet^-$	superoxide anion
OH•	hydroxyl radical
OD	optical density
PBS	phosphate buffer saline
PCR	Polymerase Chain Reaction
p-ERK	phosphorylated extracellular signal regulated kinases
PGE_2	Prostaglandin E_2

PI3K	phosphatidylinositol 3-kinase
p-JNK	phosphorylated c-Jun NH2-terminal kinase
PKB	protein kinase B
PKC	protein kinase C
PIP_2	phosphatidylinositol-(4,5)-biphosphate
PIP_3	phosphatidylinositol-(3,4,5)-triphosphate
PUFA	polyunsaturated fatty acids
Q-RT	quantitative reverse transcriptase
RNA	ribonucleic acid
ROS	reactive oxygen species
SDS	sodium dodecyl sulfate
SF-1	steroidogenic factor-1
SOD	superoxide dismutase
Sp1	specificity protein 1
SSA	sulfosalicylic acid
StAR	steroidogenic acute regulatory protein
STS	steroid sulfatase
TAE	tris-acetic acid-EDTA buffer
TEMED	NNN-N'-tetramethylethylenediamine
TF	transcription factor
TNB	5-thio-2-nitrobenzoic acid
TNF-α	tumor necrosis factor-alpha
TRITC	Tetramethyl Rhodamine Isothiocyanate
VEGF	vascular endothelial growth factor
XO	xanthine oxidase

ABSTRACT

Endometriosis, an estrogen dependent disease, is characterised by ectopic endometrial tissue outside of the uterus. Earlier work proposed *'retrograde menstruation'* as a possible cause of disease development. Despite extensive research, the underlying pathogenesis of this disease has not been completely elucidated yet. Little is known about the factors that affect the continued ectopic growth. Thus, the main aim of this study is to develop the molecular mechanism of cell proliferation in endometriosis using the human immortalized endometriotic epithelial cells (12-z cell line). This study determined estradiol, oxidative stress that induced by oxidants (H_2O_2 and menadione), and a product of oxidative stress, acrolein significantly induced 12-z cell proliferation. The proliferation was enhanced when cells were treated with both estradiol and oxidants or acrolein. An altered gene expression was also determined in this cell line where estrogen biosynthesis enzymes that include aromatase, 17β-HSD Type 1, AKR1C3 and COX-2 were significantly upregulated by those compounds. In addition, PGE_2, a potent inducer of aromatase activity and ER-α expression were also induced especially by combination of estradiol and oxidants or acrolein. Levels of aromatase enzymes were also increased significantly in ovarian endometriosis samples compared to samples without endometriosis. A *'positive feedback loop'* for estradiol synthesis in 12-z cell was demonstrated in this study. The mechanism of cell proliferation also involved both MAPK/ERK1/2 and PI3K/Akt signalling pathways. The study was extended to identify the antiproliferative effect of antioxidant N-acetylcysteine. NAC inhibited cell growth and induced apoptosis in 12-z cell, which involved mitochondrial dependent and JNK signaling pathways. NAC was also able to maintain the redox balance of GSH/GSSG that is useful to protect cells from elevated ROS. HO-1 is proposed as a potential biomarker in the pathogenesis of endometriosis as it is highly expressed in ovarian endometriosis and its expression is also induced by those compounds. There is little consensus as to the most efficacious medical or surgical approach to prevent the long-term impact of endometriosis. This study proposed NAC as a potential drug to the current treatment that available for endometriosis.

CHAPTER ONE:

GENERAL INTRODUCTION

1.1 An overview of endometriosis

The uterus is a major female reproductive organ that is located in the pelvic cavity (*Figure 1.1*). The mucous membrane lining the uterus, known as the 'endometrium', plays a major role in the development of endometriosis. 'Endometriosis', a common gynaecological disorder, is characterised by the presence outside the uterus, of functioning glands and stroma that are similar to those found in the endometrium (*Figure 1.1*) (Rogers *et al.*, 2009). To clarify the terminologies that are related to endometrium, some definitions are shown in *Table 1.1.* The disease may affect various sites, including ovaries, peritoneum, uterosacral ligaments, rectocervical region, rectovaginal septum and bladder (*Figure 1.2*) (Abrao *et al.*, 2009). Endometriosis commonly presents during reproductive age (Rizner, 2009), but it can also present in early adolescence and postmenopausal women (Amer, 2008). There are three main types of endometriosis, namely ovarian endometriosis, peritoneal endometriosis and deep endometriotic nodules (Nap *et al.*, 2004). The disease is an estrogen-dependent disease as it is known to require estrogen for its continued growth (Zeitoun *et al.*, 1998; Izawa *et al.*, 2008). Interestingly, endometriosis is also known as a progesterone-unresponsive disease (Osteen *et al.*, 2005) and progesterone resistance disease due to an overall reduction in the levels of progesterone receptors (Bulun *et al.*, 2006). Therefore, both estrogen and progesterone play a pivotal role in endometriosis.

Historically, a German physician, Daniel Shroen, described endometriosis for the first time in 1690 where he observed ulcers distributed only in the female peritoneum with inflammation and adhesions (Signorile & Baldi, 2010). The histological description was provided by Van Rokitansky in 1860 (Sharpe-Timms & Young, 2004) and later by Von Recklinghausen in 1893 (Brosens *et al.*, 1993). However, it is widely accepted that the person who named and identified the cause of the disease was John Sampson in 1921, where he observed ovarian cysts and

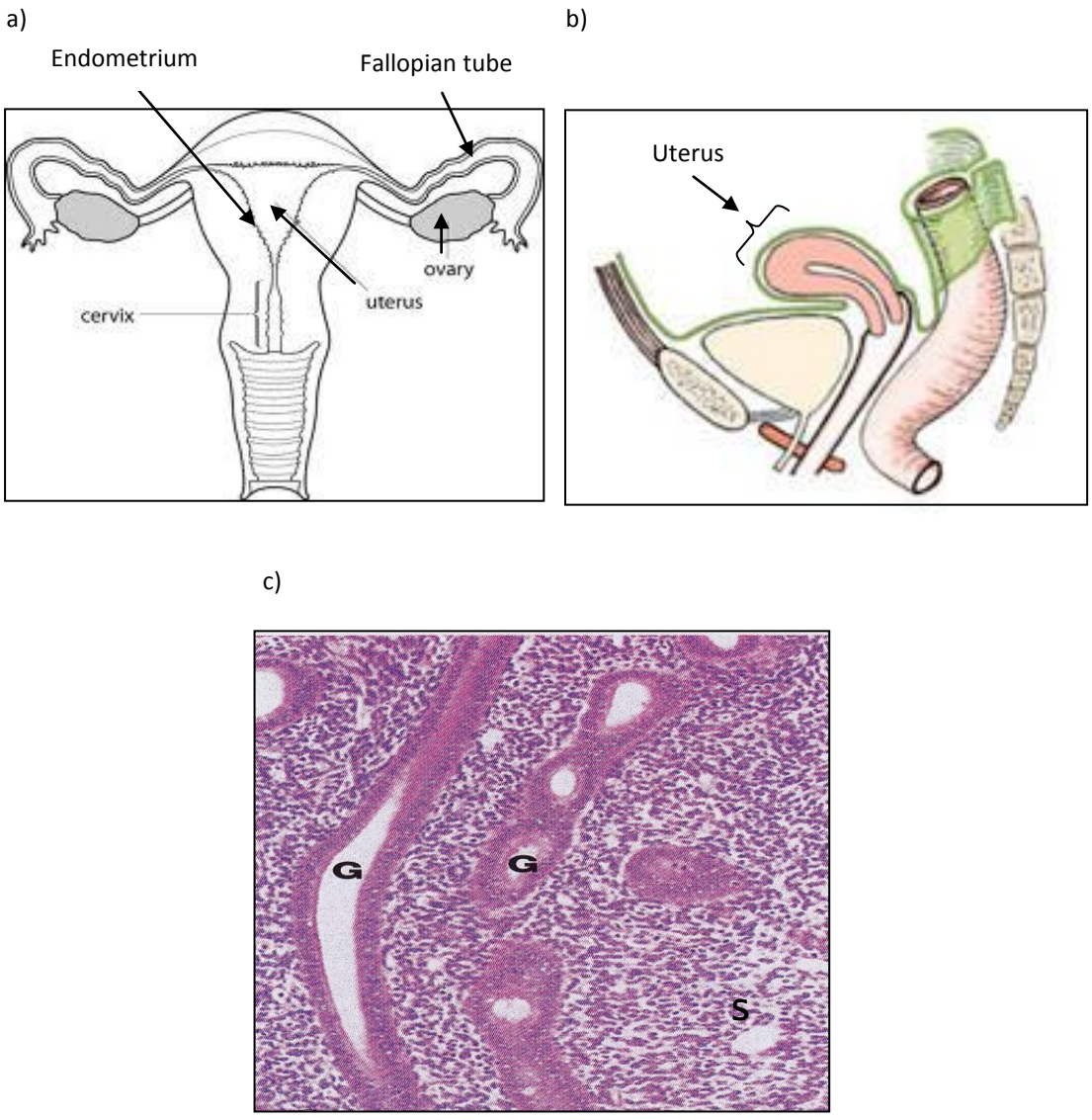


Figure 1.1: Female reproductive organs and histology of endometrium.
a) Female reproductive organs including uterus that lined by endometrium, cervix, fallopian tubes and ovaries b) Median section of female reproductive organs showing the peritoneum - a thin membrane that lines the pelvic cavity and organs c) Histology of endometrium that consists of glands (G) and stroma (S) - Adapted from (Young & Wheater, 2006).

Table 1.1: Definitions of some terminologies related to endometrium

Terminology	Definition
Ectopic	'Displacement' or abnormal location
Endometrial/endometrium	Eutopic or intrauterine endometrial tissue in its normal location
Endometriosis/Endometriotic tissue/Ectopic endometrium	Pathological ectopic endometrium-like tissues outside the uterus
Endometrioma	Endometriotic cysts of the ovary
Endometrial epithelial	Endometrial surface which is columnar in form that forming numerous simple tubular glands
Endometrial stromal	Stromal part of the endometrium that support the glands

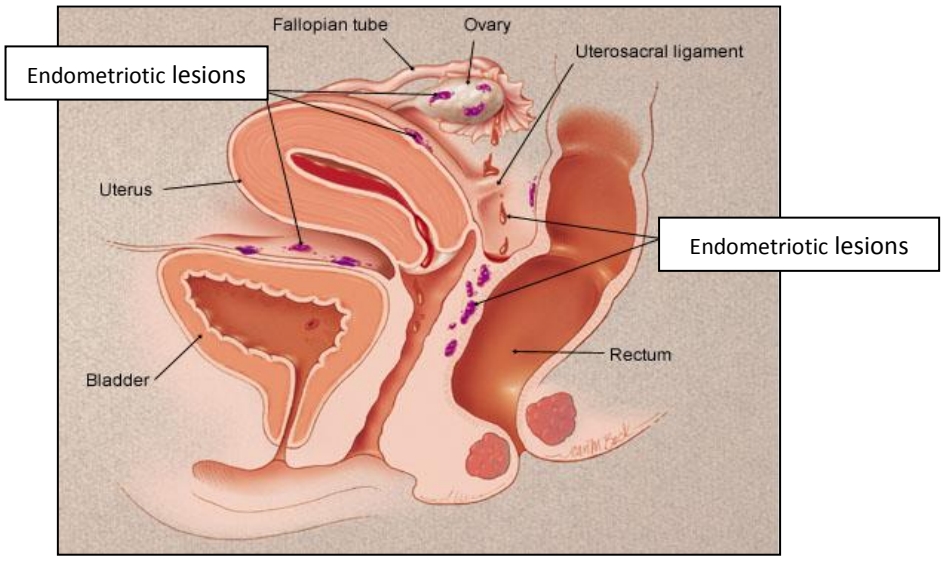


Figure 1.2: Endometriotic lesions/implants on various pelvic organs.

peritoneal spots that led him to believed that during menstruation particles of endometrium might flow backward through the tubes and implant in ovaries and peritoneum (Brosens *et al.*, 1993). This phenomenon of 'backward flow' is described further in the subsequent section of this chapter. John Sampson named the lesions as 'endometriomas' and the disease as 'endometriosis' due to its endometrial origin.

1.1.1 Epidemiology of endometriosis

The actual frequency of endometriosis in the general population is difficult to ascertain as a lot of women with endometriosis are asymptomatic, which may lead to an underestimation of the number of cases and not helped by the variability in its diagnostic methods (Flores *et al.*, 2008). As a result, many affected women remain undiagnosed. However, several authors have estimated endometriosis affect 10% of women of reproductive age, from all ethnic and social groups especially between the ages of 30 and 40 years (Vigano *et al.*, 2004; Carli *et al.*, 2009; Matalliotakis *et al.*, 2009). Other estimates of the prevalence rates of endometriosis have been reported and range from 20% to 40% in infertile women (Mahmood & Templeton, 1991; Ozkan *et al.*, 2008), 6% to 18% in women undergoing sterilization (Matorras *et al.*, 1995) and 15% to 70% in patients with chronic abdominal pain (Waller *et al.*, 1993).

1.1.2 Clinical presentations of endometriosis

Although the endometriotic cells are found in an unusual environment, they will go through the same process as the endometrium, whereby every month they grow during the menstrual cycle and these endometriotic lesions also bleed. The blood has no way of leaving the body and is trapped. The lesions are responding to cyclic secretion of estrogen and progesterone, leading to cyclic pelvic pain starting before

the menstrual period and continuing throughout the cycle until the end of the menstrual flow (Hurd, 1998; Gupta *et al.*, 2006). Matalliotakis and colleagues determined a relationship between menstrual characteristics and the development of endometriosis (Matalliotakis *et al.*, 2008). These authors demonstrated women with endometriosis have early onset of menarche, shorter menstrual cycle lengths, and heavy menstrual bleeding as compared to normal women. The pain is believed to arise from cyclical bleeding into the surrounding tissues, resulting in inflammation, formation of scarring and adhesions (Patwardhan *et al.*, 2008).

However, it has been determined recently that there is no relationship between the severity of disease and pain (Sepulcri Rde & do Amaral, 2009). Dysmenorrhoea, pain that only occurs during menstruation, is the commonest presenting symptoms affecting about 85% of women with endometriosis (Amer, 2008). It has also been reported that endometriosis-related dysmenorrhoea is usually very severe, incapacitating and unresponsive to analgesics (Amer, 2008). Other than pelvic pain, patients may present with dysparaeunia (pain during intercourse), menstrual irregularities and infertility (Valle, 2002; Kennedy *et al.*, 2005; Matalliotakis *et al.*, 2007; Flores *et al.*, 2008). The symptoms may be unusual because of the varying locations of the disease and do not always correlate with the extent of the lesions in many cases (Bedaiwy & Falcone, 2004). The associated symptoms can impact on general physical, mental and social wellbeing (Kennedy *et al.*, 2005; Rogers *et al.*, 2009). Furthermore, it has been proven recently that the disease may cause depression and anxiety due to the pain and long duration of treatment (Sepulcri Rde & do Amaral, 2009).

1.1.3 Endometriosis associated with infertility

Endometriosis is a common public health issue especially because of its association

with infertility. It has been reported that endometriosis has a prevalence of 0.5-5% in fertile and 20-40% in infertile women (Ozkan *et al.*, 2008). However, Valle has proposed 50% of infertile women have endometriosis and that a similar percentage of women with endometriosis are infertile (Valle, 2002). Although there is a reasonable body of evidence associating endometriosis with infertility, the causal relationship has not been established and it appears that infertility in endometriosis is multifactorial. The causes of infertility in endometriosis have been classified in various ways. For example, it has been divided into i) advanced endometriosis that has a mechanical mechanism including adhesions, large ovarian endometriotic cysts (endometriomas) and distortion of the tubovarian relationship and ii) mild endometriosis, in which the mechanism is incompletely understood, including peritoneal fluid abnormalities, ovulatory dysfunction, immunological abnormalities, follicular and oocyte abnormalities, and embryonic implantation failure (Amer, 2008).

The peritoneal fluid of women with endometriosis has long been the focus of investigation as a mediator of infertility in endometriosis (Ryan & Taylor, 1997; Mahutte & Arici, 2002; Noordin *et al.*, 2008). Due to a direct communication between the peritoneal fluid and fallopian tubes, it is presumed that oocytes, spermatozoa and embryos are exposed to the peritoneal fluid and its cellular and soluble components. Numerous investigators proposed that the toxic substances in the peritoneal fluid are produced by macrophages and may affect reproductive function at various levels. Macrophages have been demonstrated to secrete various substances such as cytokines, growth factors, prostanoids, complement components and hydrolytic enzymes (Nathan, 1987). Among these products, cytokines have attracted numerous researchers to identify the mechanism of endometriosis associated infertility. Cytokines may affect gamete function (Ramey & Archer, 1993), fertilization (Sueldo *et al.*, 1987), implantation (Harada *et al.*, 2001), embryo development (Ramey & Archer, 1993; Noordin *et al.*, 2008) and post

implantation survival (Oral *et al.*, 1996). In addition, as ovaries are also bathed in the peritoneal fluid, thus oocytes are exposed to the peritoneal environment even after they are captured by the fimbria of the Fallopian tube because the tube is a conduit freely communicating with the peritoneal fluid (Oral *et al.*, 1996).

1.1.4 Diagnosis of endometriosis

In order to make a diagnosis for any disease, a detailed history-taking is required to identify relevant symptoms. The presentation of endometriosis is variable, which may overlap with other conditions such as irritable bowel syndrome and pelvic inflammatory disease (Kennedy *et al.*, 2005). It is crucial however, to perform clinical examination especially in the diagnosis of pelvic endometriosis including abdominovaginal examination as lesions implanted in the retrocervical area or rectovaginal wall are frequently more easily felt than seen (Brosens *et al.*, 1993). Laparoscopy has been widely considered to be the gold standard tool for the diagnosis as it provides direct visualization of endometriotic lesions and allows staging of the disease in terms of extent of adhesions, number and size of lesions (Kennedy *et al.*, 2005; O'Callaghan, 2006). The endometriotic cells from the uterus detach and invade the pelvic cavity and attach anywhere on the peritoneal surface. They may grow and invade the reproductive organs, adjacent pelvic structures, and other parts of the body. Morphologically, endometriosis is diverse. The endometriotic implants may be cystic, fibrotic or inflamed, and their colour may be red, yellow, brown or black, whereas histologically, they are similar to endometrium with addition of hemorrhage, inflammatory cells and fibrous connective tissue (Abrao *et al.*, 2003). Thus, to diagnose endometriosis accurately, a combination of non-invasive and invasive procedures are required (Rogers *et al.*, 2009).

1.1.5 Classification of endometriosis

To provide standardization of classification of endometriosis, multiple classification systems were proposed. However, most of the classification systems proposed led to confusion and no single system gained universal acceptance. This led the American Fertility Society to design a standard classification system, which has become widely accepted by many authors as the standard classification scheme, as shown in *Figure 1.3, 1.4 and 1.5*.

1.1.6 Treatments available for endometriosis

The goals of treatment in endometriosis are to destroy or remove most or all of the implants, restore the normal anatomy, prevent or delay progression, and relieve the patient's symptoms (Bulletti *et al.*, 2001; Valle, 2002). Several management options are available to treat endometriosis including analgesic, hormonal treatment and surgery. To date, no medical therapy that is effective in eradicating the disease or in preventing it without unacceptable side effects. Furthermore there is little consensus as to the most efficacious medical or surgical approach for limiting the disease progression. Surgery offers a more definitive treatment that can achieve long-term control of painful symptoms and is proven to improve fertility (Amer, 2008). In addition, this author reported that the incidence of disease recurrence at five-year follow-up is about 20% for surgery compared to about 50% for medical treatment. However, others have shown the recurrence of endometriosis is about 40% of patients after surgical removal (Bulletti *et al.*, 2001). Despite ongoing improvement in the treatment of endometriosis, the disease still awaits optimal therapy as high recurrence rates are still reported. Although several management options are available, the choice of treatment should be tailored to individual circumstances (Kennedy *et al.*, 2005).

AMERICAN FERTILITY SOCIETY CLASSIFICATION OF ENDOMETRIOSIS

Patient's name: _____

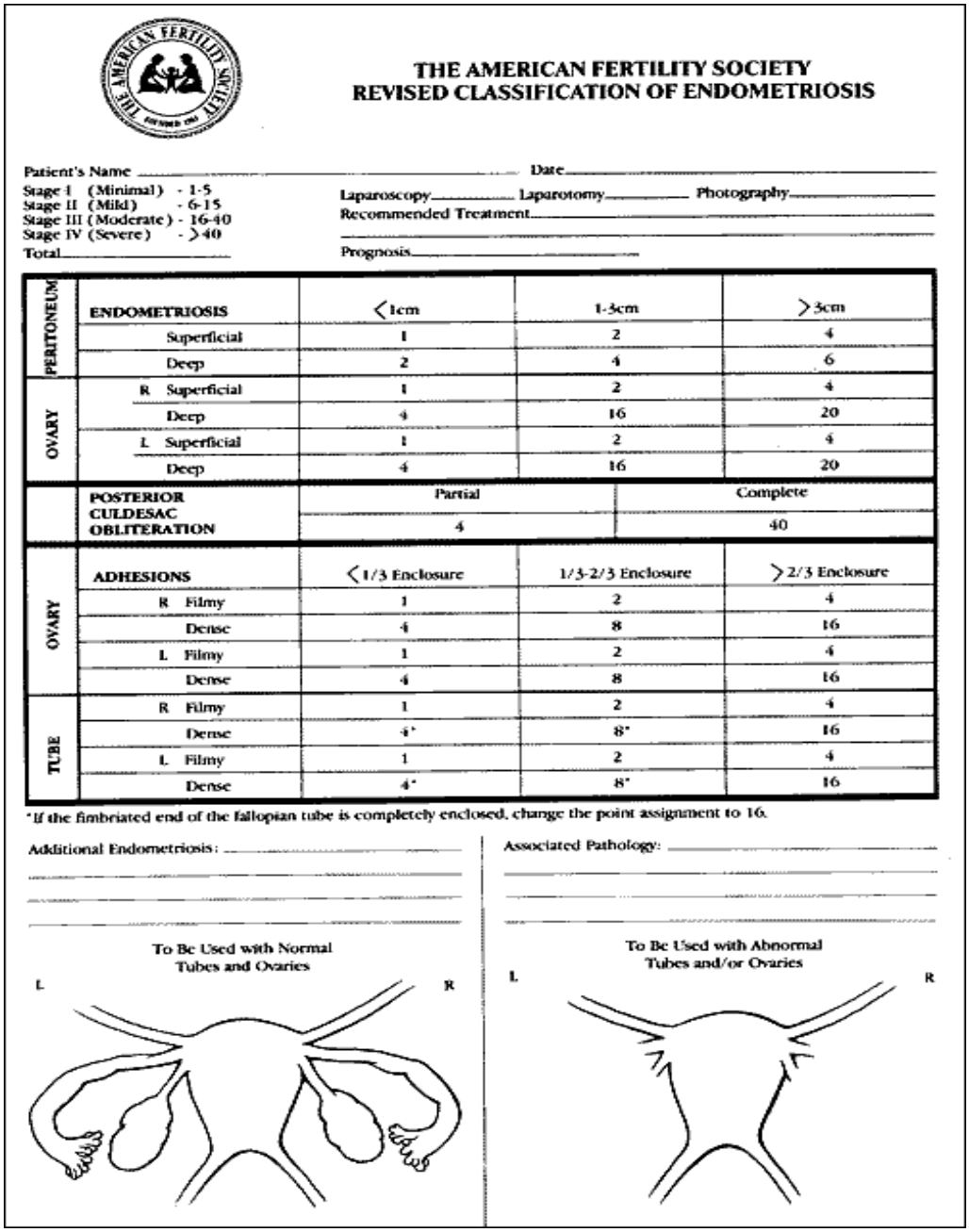
Stage I	**(Mild)**	**1-5**
Stage II	**(Moderate)**	**6-15**
Stage III	**(Severe)**	**16-30**
Stage IV	**(Extensive**	**31-54**

Total: _____

PERITONEUM	**ENDOMETRIOSIS**	**<1cm**	**1-3cm**	**>3cm**
		1	**2**	**3**
	ADHESIONS	**filmy**	**dense w/partial cul-de-sac obliteration**	**dense w/complete cul-de-sac obliteration**
		1	**2**	**3**
OVARY	**ENDOMETRIOSIS**	**<1cm**	**1-3cm**	**>3cm or ruptured endometriome**
	R	**2**	**4**	**6**
	L	**2**	**4**	**6**
	ADHESIONS	**filmy**	**dense w/partial ovarian enclosure**	**dense w/complete ovarian enclosure**
	R	**2**	**4**	**6**
	L	**2**	**4**	**6**
TUBE	**ENDOMETRIOSIS**	**<1cm**	**>1cm**	**tubal occlusion**
	R	**2**	**4**	**6**
	L	**2**	**4**	**6**
	ADHESIONS	**filmy**	**dense w/tubal distortion**	**dense w/tubal enclosure**
	R	**2**	**4**	**6**
	L	**2**	**4**	**6**

Associated Pathology

Figure 1.3: The original American Fertility Society Classification, 1979. This classification has divided endometriosis into 4 stages, based on the total scores of each category as above (AFS, 1979).



THE AMERICAN FERTILITY SOCIETY
REVISED CLASSIFICATION OF ENDOMETRIOSIS

Patient's Name _____ Date _____

Stage I (Minimal) - 1-5
Stage II (Mild) - 6-15
Stage III (Moderate) - 16-40
Stage IV (Severe) - >40
Total _____

Laparoscopy _____ Laparotomy _____ Photography _____
Recommended Treatment _____
Prognosis _____

	ENDOMETRIOSIS	<1cm	1-3cm	>3cm
PERITONEUM	Superficial	1	2	4
	Deep	2	4	6
OVARY	R Superficial	1	2	4
	Deep	4	16	20
	L Superficial	1	2	4
	Deep	4	16	20

POSTERIOR CULDESAC OBLITERATION	Partial	Complete
	4	40

	ADHESIONS	<1/3 Enclosure	1/3-2/3 Enclosure	>2/3 Enclosure
OVARY	R Filmy	1	2	4
	Dense	4	8	16
	L Filmy	1	2	4
	Dense	4	8	16
TUBE	R Filmy	1	2	4
	Dense	4*	8*	16
	L Filmy	1	2	4
	Dense	4*	8*	16

*If the fimbriated end of the fallopian tube is completely enclosed, change the point assignment to 16.

Additional Endometriosis: _____

Associated Pathology: _____

To Be Used with Normal Tubes and Ovaries

L R

To Be Used with Abnormal Tubes and/or Ovaries

L R

Figure 1.4: Revised American Fertility Society classification of endometriosis, 1985. Some modifications have been made to the original classification, which differentiate between superficial and invasive nature of endometriosis (AFS, 1985).
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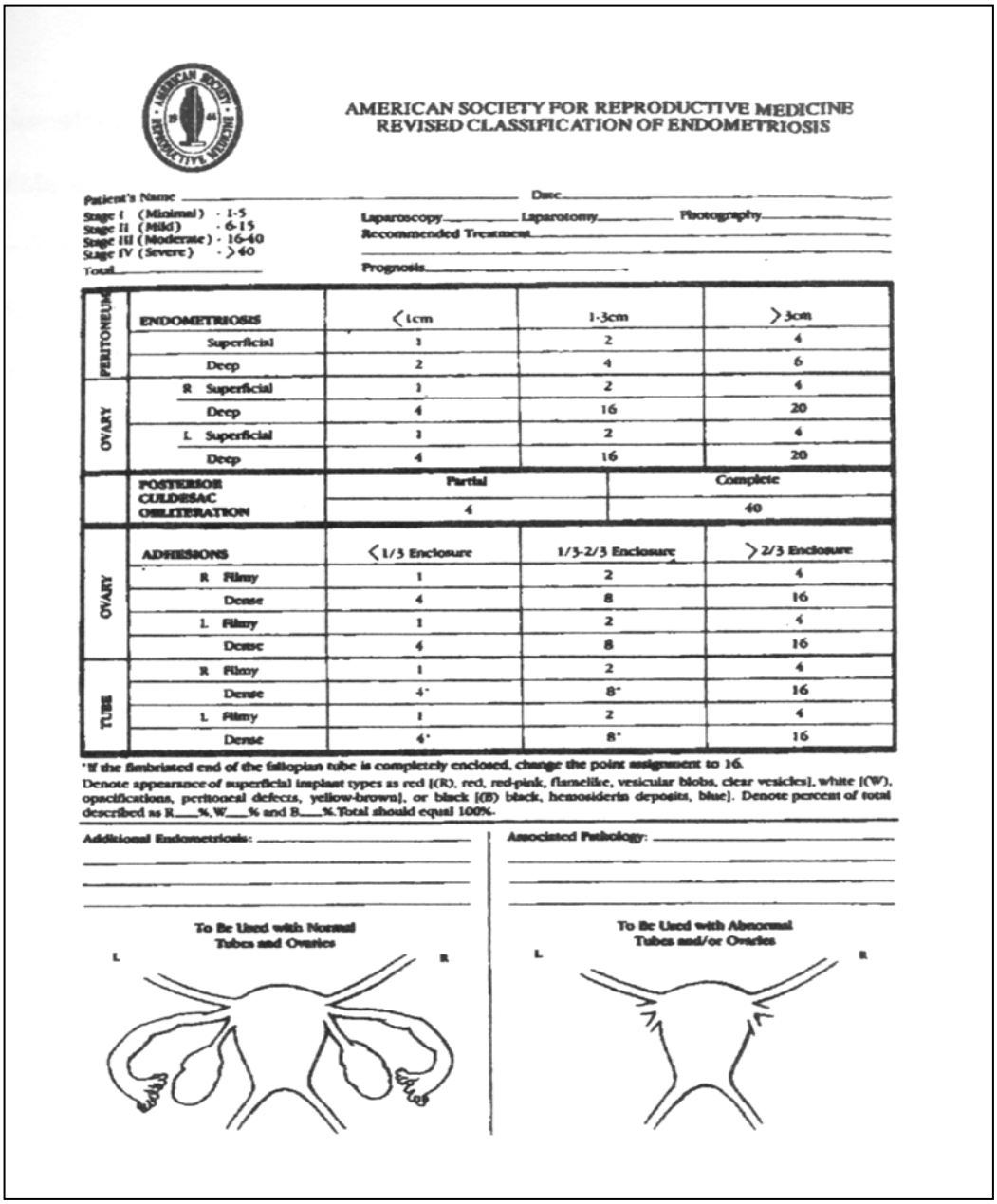


AMERICAN SOCIETY FOR REPRODUCTIVE MEDICINE
REVISED CLASSIFICATION OF ENDOMETRIOSIS

Patient's Name _____ Date _____

Stage I (Minimal) - 1-5
Stage II (Mild) - 6-15
Stage III (Moderate) - 16-40
Stage IV (Severe) - >40
Total _____

Laparoscopy _____ Laparotomy _____ Photography _____
Recommended Treatment _____
Prognosis _____

		<1cm	1-3cm	>3cm
PERITONEUM	ENDOMETRIOSIS			
	Superficial	1	2	4
	Deep	2	4	6
OVARY	R Superficial	1	2	4
	Deep	4	16	20
	L Superficial	1	2	4
	Deep	4	16	20
	POSTERIOR CULDESAC OBLITERATION	Partial		Complete
		4		40
OVARY	ADHESIONS	<1/3 Enclosure	1/3-2/3 Enclosure	>2/3 Enclosure
	R Filmy	1	2	4
	Dense	4	8	16
	L Filmy	1	2	4
	Dense	4	8	16
TUBE	R Filmy	1	2	4
	Dense	4*	8*	16
	L Filmy	1	2	4
	Dense	4*	8*	16

*If the fimbriated end of the fallopian tube is completely enclosed, change the point assignment to 16.

Denote appearance of superficial implant types as red [(R), red, red-pink, flamelike, vesicular blobs, clear vesicles], white [(W), opacifications, peritoneal defects, yellow-brown], or black [(B) black, hemosiderin deposits, blue]. Denote percent of total described as R____%, W____% and B____%. Total should equal 100%.

Additional Endometriosis: _____

Associated Pathology: _____

To Be Used with Normal Tubes and Ovaries

L R

To Be Used with Abnormal Tubes and/or Ovaries

L R

Figure 1.5: Revised American Society for Reproductive Medicine classification of endometriosis, 1996. Further modifications were made by adding the appearance of the endometrial implants (ASRM, 1997).

It is also recommended that the best treatment for endometriosis is generally surgery combined with pharmacotherapy (Halis *et al.*, 2010). Since there is no cure for endometriosis at present, treatment is aimed at reducing symptoms, achieving pregnancy in case of infertility, and preventing pain and progression of the disease. The European Society of Human Reproduction and Embryology (ESHRE) Guidelines is among the professional guidelines that have been widely used to treat endometriosis. ESHRE guidelines for the management of endometriosis, state that good clinical practice should include offers of counselling, analgaesia and nutritional therapy combined with progestogens, the combined oral contraceptives (COCP) or gonadotrophin releasing hormone agonists (GnRH) analogues, as an empirical treatment for pelvic pain presumed to be endometriosis (Kennedy *et al.*, 2005). In addition, during laparoscopy, ideal clinical practise should be to surgically remove any endometriotic lesions at the same time.

The Practice Committee of the American Society for Reproductive Medicine (2008), recommended that endometriosis should be viewed as a chronic disease that requires a life-long management plan with the goal of maximizing the use of medical treatment and avoiding repeated surgical procedures (ASRM, 2008). Most recently, Vercellini and colleagues have proposed the pharmacological therapy must achieve two main objectives; relief of pain for prolonged periods and prevention of disease progression (Vercellini *et al.*, 2011).

In 2003, a systematic guideline of medical management of endometriosis has been proposed as below (Mahutte & Arici, 2003):

First Line Medical Treatments

- *Oral Contraceptives*-The combined oral contraceptive pills (COCPs) estrogens and progestogens (e.g ethinyl estradiol plus progestin) have been shown to reduce menstrual blood flow and decidualization of the ectopic

endometrium with decreased cell proliferation and increased apoptosis (Meresman *et al.*, 2002). Decidualization is a characteristic of the endometrium of the pregnant uterus in response to progesterone.

- *Non steroidal anti-inflammatory drugs (NSAIDs)* (e.g Naproxen, ibuprofen and mefenamic acid) - NSAIDs are drugs that act by blocking prostaglandin production that is well known to cause abdominal pain. NSAIDs were found significantly more effective for abdominal pain relief as compared to placebo or paracetamol (Marjoribanks *et al.*, 2010).

Second line Medical Treatments

- Progestins (e.g Dienogest, Dydrogesterone, Gestrinone and Norethindrone acetate) - Progesterone only drugs are the most popular medical treatment of endometriosis for more than 40 years (Schweppe, 2001). These drugs are able to suppress ovulation and reduce serum estrogen levels (Luciano *et al.*, 1988). In addition, previous *in vitro* studies have demonstrated Dienogest has anti-proliferative effects by directly inhibited human endometrial epithelial cell growth (Shimizu *et al.*, 2009).

Third line Medical Treatments

- Gonadotropin-releasing hormone agonists analogues or GnRH analogues (e.g Leuprolide, Leuprolide depot and Triptorelin) - GnRH analogues are modified forms of GnRH that bind to GnRH receptors. Due to their longer half life than native GnRH (Huang, 2008), these agents have been reported to downregulate GnRH receptors, thus causing decreased gonadotrophin secretion, suppression of ovulation and reduced serum levels of estrogen (Valle & Sciarra, 2003). However, long term use of GnRH is associated with substantial bone mineral density (BMD) loss with 3.2% reduction in lumbar spine BMD after 6 months of treatment that limits the use of these agents to a maximum of 6 months duration (Child & Tan, 2001). Therefore, the use of 'add-back' therapy was introduced to prevent or reduce the loss of BMD

that allow the longer time for using GnRH for example addition of progestin only or a progestin with estrogen (Child & Tan, 2001).

Fourth line Medical Treatments

- In the case of no improvement with the above medications or if side effects are too pronounced, repeat surgery is warranted.

In addition to the above drugs, Danazol (a synthetic androgen) is also used to treat endometriosis. This drug is able to produce a high androgen and low estrogen environment that results in atrophy of the endometriotic implants (Huang, 2008). However, the occurrence of androgenic side effects such as weight gain, breast atrophy and hirsutism (Huang, 2008), make the use of this drug very limited. Aromatase inhibitors (AIs) have also been shown to have potential effects in the treatment of endometriosis. Aromatase is a key enzyme for estrogen biosynthesis and further details about this enzyme are described in section 1.4.1.1.1. The AIs are classified into type I inhibitors (non-competitive - e.g Exemestane) that irreversibly binds to the enzyme and inactivates it, and type II (competitive inhibitor - e.g Anastrozole and Letrozole) that compete with androgen precursor for the enzyme (Attar & Bulun, 2006b). Both types of inhibitors are for binding to the active site of aromatase enzyme. These drugs have succesfully reduced the pelvic pain and the size of endometriotic lesions (Ailawadi *et al.*, 2004; Razzi *et al.*, 2004). However, the aromatase inhibitors that block estrogen biosynthesis by the aromatase enzyme in endometriotic implants need to be used with second agents such as oral contraceptives or GnRH analogues to suppress ovarian function. For example, previous studies have demonstrated the combination of an aromatase inhibitor (letrozole) and progestin (norethindrone) for 6 months resulted in was able to reduce pain symptoms and absence of endometriotic lesions on the second-look laparoscopy (Ailawadi *et al.*, 2004).

Unfortunately, despite adequate medical and/or surgical treatment, pain symptoms may persist, which require a multi-disciplinary approach such as involving a pain clinic and counselling (Kennedy *et al.*, 2005).

1.2 Endometrium

As mentioned earlier, endometrium is the mucous membrane lining of the uterus plays a major role in the development of endometriosis.

1.2.1 Histology of the endometrium

The endometrium consists of a single layer of columnar epithelium forming numerous tubular glands supported by a layer of connective tissue, stroma as shown in *Figure 1.1*.

Functionally, the endometrium is divided into two layers;

i. The functional layer: is a thick superficial layer, consists of few glands and abundant stroma. It is highly responsive to hormonal ovarian influence that induces the sequential glandular and stromal changes of the menstrual cycle.

ii. The basal layer: this layer gives rise to the new grows of functional layer. It is adjacent to the myometrium just below the functional layer, which consists of many glands and less stroma. The basalis corresponds to approximately the lower one third of the endometrium and retains essentially identical histologic features throughout the menstrual cycle.

1.2.2 Changes in the endometrium in response to steroid hormones

During the reproductive years, the endometrium undergoes regular cyclic changes, namely menstrual cycle for 28 days, as a response to the release of the ovarian hormones, estrogen and progesterone, as shown in *Figure 1.6*. Three phases of the menstrual cycle are recognized; i. *Menstrual phase*: the functional layer of the endometrium becomes detached from the uterine wall and these results in bleeding ii. *Proliferative phase*: as plasma levels of estrogen increase the endometrium begins to proliferate and thicken, tubular glands and spiral arteries form iii. *Secretory phase*: rising levels of progesterone results in stimulating the enlargement of glands and secreting mucus and glycogen. In addition, progesterone inhibits the proliferative activity of the endometrium and induces secretory activity (Deligdisch, 2000). These cycles of cells growth/death is also known as tissue remodelling (Mor *et al.*, 2002). The human endometrium exhibits these periodic changes in response to the rise and fall of ovarian steroids including estrogen and progesterone. The endometriotic tissues respond to the menstrual hormonal changes and behave like the menstruating endometrium (Hastings & Fazleabas, 2006). Gonadotropin-releasing hormone (GnRH) is responsible for the release of Follicle stimulating Hormone (FSH) and Luteinizing hormone (LH) from the anterior pituitary. These hormones are responsible for the development of ovarian follicles. As the follicle grows, estrogen is released. FSH stimulates ovarian steroidogenesis, particularly estrogen and the rapid rise of estrogen has a negative effect on FSH. LH is mainly involved in promoting ovulation.

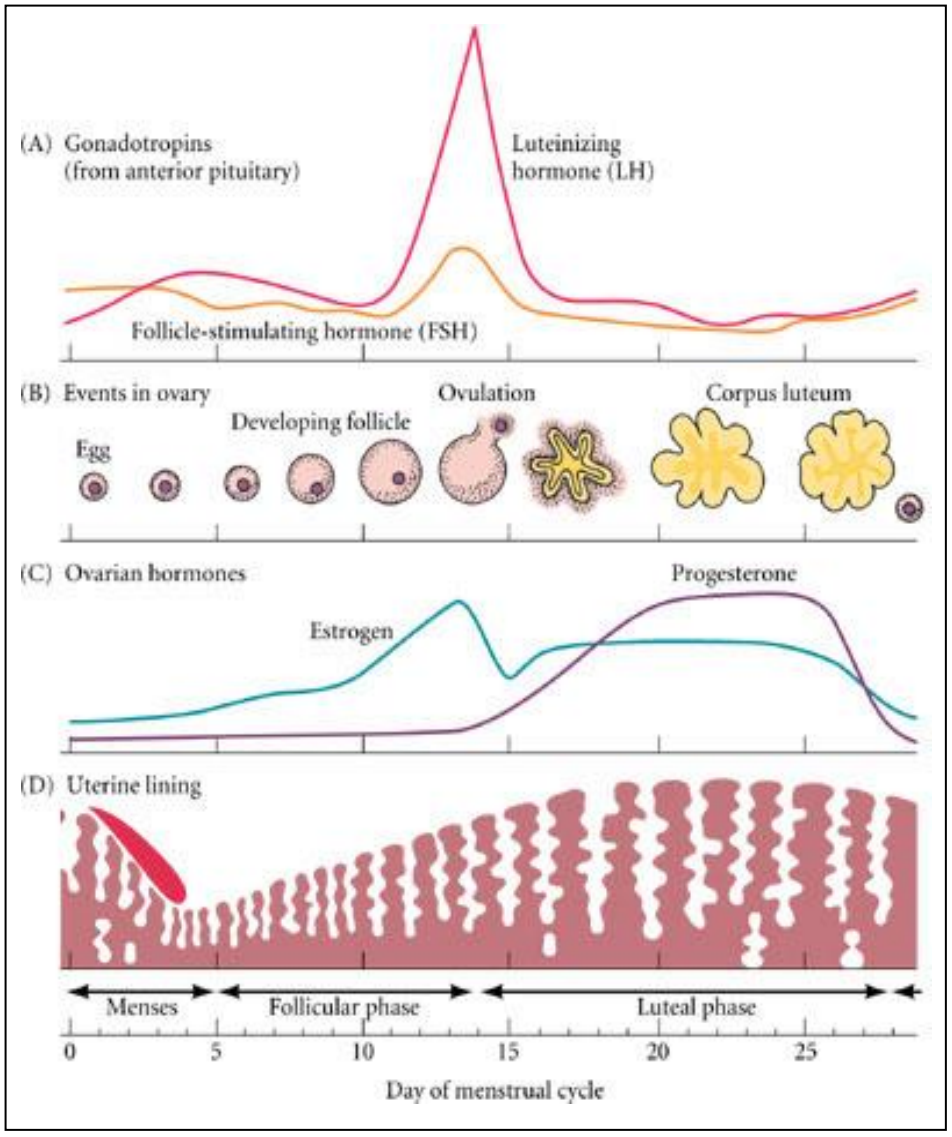


Figure 1.6: Changes in the endometrium during menstrual cycle. Three phases of menstrual cycle are shown; *Menstrual phase/menses* - The functional layer of the endometrium detach from the uterine wall and bleed. *Proliferative/follicular phase* - The endometrium begins to proliferate and thicken as levels of estrogen increase. *Secretory/luteal phase* - The rising levels of progesterone stimulate the enlargement of glands which secrete mucus and glycogen.

1.3 Theories of the origin of endometriosis

While the existence of endometriosis has been known for more than one hundred years ago, the origin of this disease remains unclear. There is no single theory can explain all types and anatomical distribution of the endometriotic lesions. How endometriosis gives rise to the ectopic lesions is not well understood, mostly because the initial stages of the disease are neither detectable nor observable in humans. There are four main theories have been postulated to explain the initiation of endometriosis.

- Retrograde menstruation
- Coelomic metaplasia theory
- Lymphatic and vascular dissemination theory
- Genetic theory

1.3.1 Retrograde menstruation

Although multiple theories have been put forth to explain the pathogenesis of this enigmatic disorder, the '*retrograde menstruation*' theory is the most popular and widely accepted. This theory was proposed by John Sampson in 1921 (Brosens *et al.*, 1993; Brosens & Brosens, 2000). According to this theory, fragments of endometrium are refluxed through the fallopian tubes (*Figure 1.7*) into the peritoneal cavity, which attach and grow on peritoneal surfaces, ovaries and ligaments, as shown earlier in *Figure 1.2*. That is, instead of draining out of the body through the vagina, the theory holds that some of the menstrual fluid backs up the fallopian tubes and drips into the pelvic cavity.

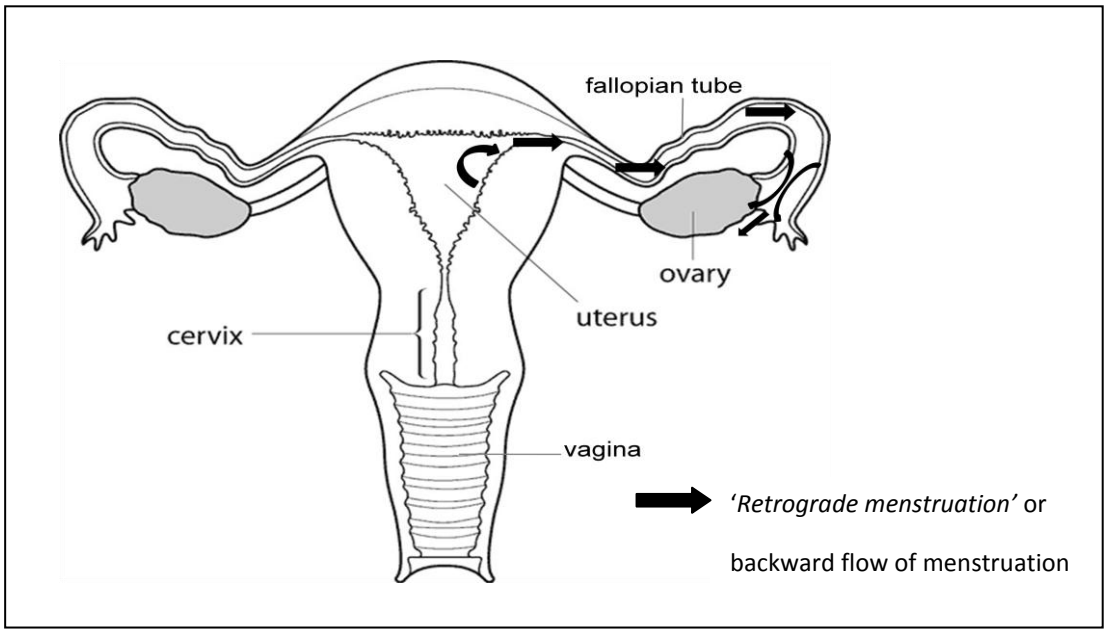


Figure 1.7: The diagram of '*retrograde menstruation*' leading to endometriotic lesions deposition.

The anatomical distribution of endometriotic lesions that is in the abdomen and more often in the pelvis may support this theory (Bricou *et al.*, 2008). In addition, Sampson's theory is further supported by the fact that retrograde menstruation through the fallopian tubes into the peritoneal fluid is a universal phenomenon in women with normal or unobstructed fallopian tubes (Halme *et al.*, 1984). The reason as to why some women develop endometriosis while others do not remains unanswered. However, it has been postulated that in normal women, the endometrial cells and tissue that arrive in the peritoneal cavity during menstruation, are effectively eradicated by the peritoneal macrophages (Santanam *et al.*, 2002). The peritoneal environment of most women is capable of resorbing the endometrial tissue present at the end of menstruation. However, this system of cleansing is inefficient or overwhelmed in patients with endometriosis (Vinatier *et al.*, 2001). Since retrograde menstruation is a very common phenomenon among women of reproductive age, there must be other factors that may contribute to the pathophysiology and/or pathogenesis of endometriosis.

Although the '*retrograde menstruation theory*' is widely accepted, little is known about the events of cell adhesion and proliferation, which ultimately result in endometriosis. The endometrial cells are able to attach to, and invade the peritoneum, and proliferate to create and maintain an endometriotic disease. Therefore, following reflux, the fragments of endometrium undergo adhesion, proteolysis, proliferation, angiogenesis and scarring (Vinatier *et al.*, 2001). Therefore, this theory requires three steps; i) retrograde menstruation has to occur ii) retrograde menstruation should contain endometrial cells and iii) adhesion to the peritoneum has to occur with subsequent implantation and proliferation (Konno *et al.*, 2007). Since retrograde menstruation occurs in most reproductive age women, it is clear that there must be certain factors in some women which contribute to the implantation and proliferation of the endometrial cells and their subsequent development into the endometriotic disease.

Although many studies have been carried out to determine the pathogenesis of endometriosis, results are rather conflicting. To date, the mechanisms whereby endometriotic lesions establish, progress and migrate are not well understood. However, Flores et al. have proposed mechanism of the establishment of this disease following retrograde menstruation as shown in (*Figure 1.8*) (Flores *et al.*, 2007).

1.3.2 Coelomic metaplasia theory

'Coelomic metaplasia theory' or Meyer's theory is the oldest theory that was proposed by Iwanoff and Robert Meyer in 1919 (Ramey & Archer, 1993). They suggested that the coelomic epithelium that forms structures such as the peritoneum, ovarian epithelium and the mullerian systems, can undergo changes to revert back to its primitive tissue of origin and then transform into

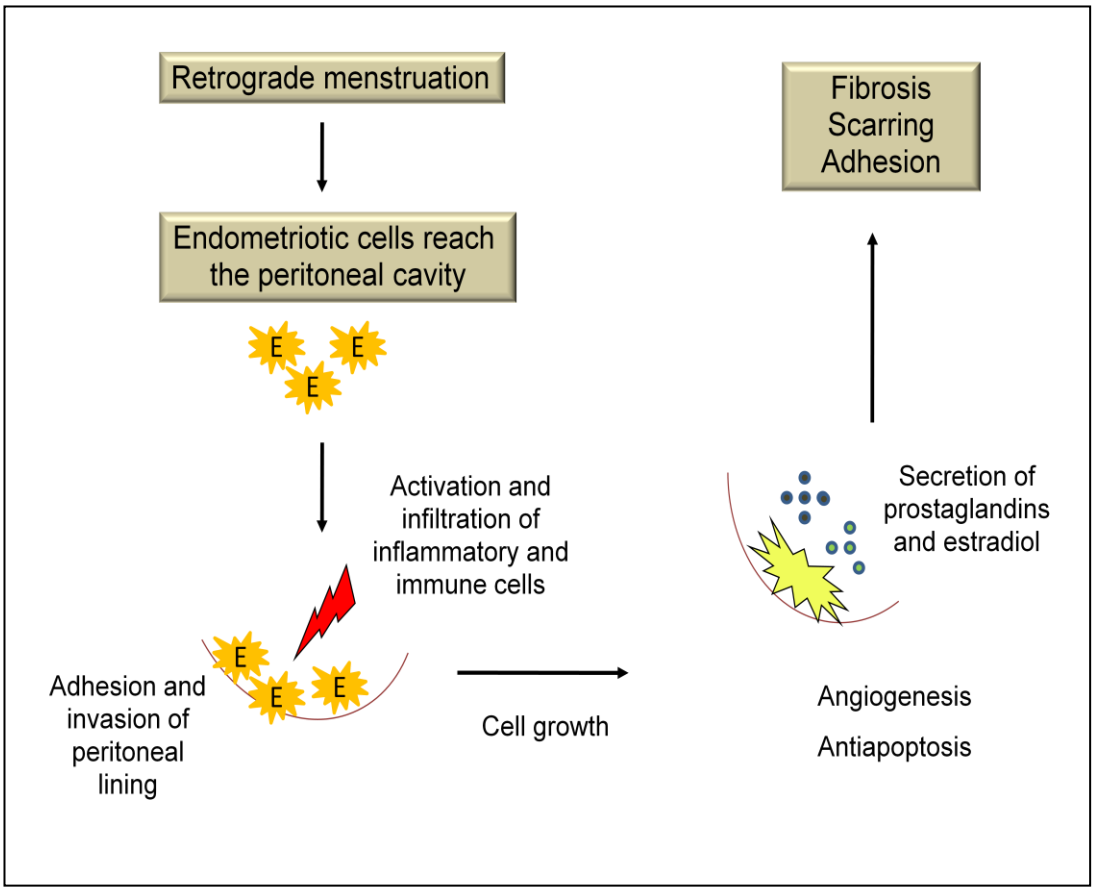


Figure 1.8: A proposed mechanism of the establishment of endometriosis. Cells reach the peritoneal cavity following retrograde menstruation, attached and invade the peritoneum. Activation of inflammatory and immune cells help the endometriotic cells to grow. The endometriotic cells, which are resistant to apoptosis will survive due to the process of angiogenesis. The secretion of prostaglandin and estradiol further increased the cell growth. In longer duration, wound healing is activated, which may result in the fibrosis, scarring and adhesion. Adapted from (Flores *et al.*, 2007).

endometriotic deposits via a process of metaplasia. It has been reported that at puberty, increases in estrogen production induce these mature peritoneal or ovarian surface cells to undergo metaplasia into endometriotic cells (Amer, 2008). This theory could explain the lesions in the abdomen and pelvis. A question that remains to be answered concerning this theory is, if peritoneal epithelium has the potential to undergo metaplasia, this phenomenon would also be expected to occur in men, but endometriosis remains the disease of the female. Unfortunately, little experimental and clinical data to support this theory exists.

1.3.3 Lymphatic and vascular dissemination theory

'Lymphatic and vascular dissemination theory' or Halban's theory was proposed by Halban in 1924. He postulated that viable endometrial cells enter the lymphatic circulation and blood vessels and embolize to ectopic sites (Amer, 2008). Javert, in 1949 further supported this theory, where he demonstrated microscopic evidence of endometrial cells in lymphatics and nodes (Valle, 2002). Endometriosis involving the ovary was suggested due to lymphatic flow from the uterus to the ovary (Ueki, 1991). This theory probably can explain the rare deposits in distant sites such as lungs and diaphragm (Valle, 2002). Similar to '*coelomic metaplasia theory'*, there is lack of convincing experimental evidence to support this theory.

1.3.4 The genetic theory

'The genetic theory' proposes genetic factors that influence the susceptibility of some women to endometriosis (Kashima *et al.*, 2004). The risk of first degree relatives to develop endometriosis is higher (Lamb *et al.*, 1986) where women with endometriosis are more likely to have a mother or sister with the disease (Cramer & Missmer, 2002); about 4 to 8 times that of the general population (Stefansson *et al.*, 2002) and the incidence is further increased in the case of monozygotic twins

(Hadfield *et al.*, 1997). Several genetic abnormalities or mutations have been suggested that might be related to endometriosis, including galactose-1-phosphate uridyl transferase (GALT), microsomal cytochromes P4501A1 and 19 (CYP1A1, CYP19), glutathione-S-transferase M1 and T1 gene (GSTM T-1) (Arvanitis *et al.*, 2003), and N-acetyltransferase 2 gene (NAT-2) (Nakago *et al.*, 2001). It is possible that an interaction between genetic factors and aspects of other theories may be responsible for the occurrence of the disease.

1.4 Factors promoting progression of endometriosis

Indeed, whatever the origin of endometriotic cells, the cause that stimulates their growth and proliferation is of much interest. Previously, Banu and colleagues have proposed endometriotic cells have certain atypical properties that include; i) steroidogenic potential ii) continuous proliferation and resistance to apoptosis iii) promoting neoangiogenesis iv) migrating and invading, and v) modulating the local immune system (Banu *et al.*, 2008). Furthermore, endometriosis is believed to be caused by molecular defects of the cell that favor survival and proliferation of endometrial tissue in menstrual debris that reach the peritoneum (Bulun *et al.*, 2010). With regards to the factors promoting endometriotic cell survival and proliferation, many factors have been implicated in the pathogenesis of endometriosis including estrogen (Zeitoun *et al.*, 1998; Izawa *et al.*, 2008; Bulun *et al.*, 2010), elevated reactive oxygen species (ROS) (Agarwal *et al.*, 2003), neoangiogenesis (Flores *et al.*, 2007), integrin (Klemmt *et al.*, 2007) and much more. In this study, we are interested to determine roles of estrogen and ROS as factors responsible in the mechanism of endometriotic cell proliferation.

1.4.1 Roles of estrogen in endometriosis

Estrogens are steroidal hormones that are synthesised by the ovary and several other tissues. Steroids are bound to carrier proteins, which deliver them to their target cells. Upon reaching the target cells, steroid hormones traverse the plasma membrane by free diffusion (Lin & Scanlan, 2005). Estrogens regulate many physiological processes, including growth, differentiation, maturation and function of a wide variety of reproductive and nonreproductive tissues (Katzenellenbogen *et al.*, 2000). Bone, liver, brain and cardiovascular system are among the non reproductive tissues that affected by estrogens (Katzenellenbogen, 1996). There are three forms of circulating estrogen; estrone (E1), estradiol (E2) and estriol (E3), the most potent of which is E2 (17B-estradiol). The chemical structures of each type of estrogen that is composed of four fused rings are shown in *Figure 1.9*.

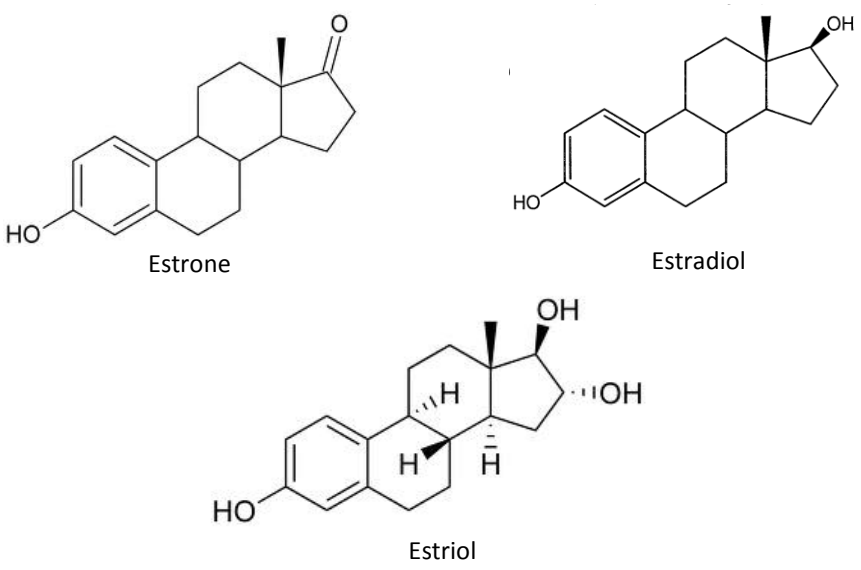


Figure 1.9: Structures of estrone (E1), estradiol (E2) and estriol (E3). Each estrogen is composed of four fused rings.

1.4.1.1 Estrogen biosynthesis and metabolising enzymes

Estrogen is produced from cholesterol that facilitated by the steroidogenic acute regulatory (StAR) protein in theca interna cells in the ovary to enter the mitochondrion. It has been shown that the endometriotic tissues can synthesise E2 *de novo* from cholesterol (Attar & Bulun, 2006a). Endometriotic tissues show high E2 biosynthesis and low E2 inactivation when compared with normal endometrium (Attar & Bulun, 2006a; Izawa *et al.*, 2008). However to date, the precise molecular basis of estrogen action on cellular proliferation in endometriosis is still unclear. Multiple estrogen biosynthesis and metabolising enzymes have been implicated in the pathogenesis of endometriosis as below.

1.4.1.1.1 Aromatase

Aromatase is a member of the cytochrome P450 (CYP) superfamily, which is encoded by the CYP19A1 gene on chromosome 15q21.1. It consists of a haem group and a polypeptide chain of 503 amino-acid residues (Ghosh *et al.*, 2009). The crystal structure of human aromatase is shown in *Figure 1.10*. This enzyme is expressed in several tissues including breast, ovary, endometrium, placenta, testes, skin, bone, fat and brain (Vercellini *et al.*, 2011). Aromatase is a well known key enzyme for estrogen biosynthesis as it catalyses the conversion of androgen (C_{19}), namely androstenedione and testosterone, to estrone and estradiol (E2) respectively (Delvoux *et al.*, 2009; Rizner, 2009). The local biosynthesis of estrogen pathways is shown in *Figure 1.11*. As the key enzyme for estrogen biosynthesis, this enzyme has been the focus of investigation for many years to determine the mechanism of endometriotic cell proliferation and factors that influence its expression and activity have been studied.

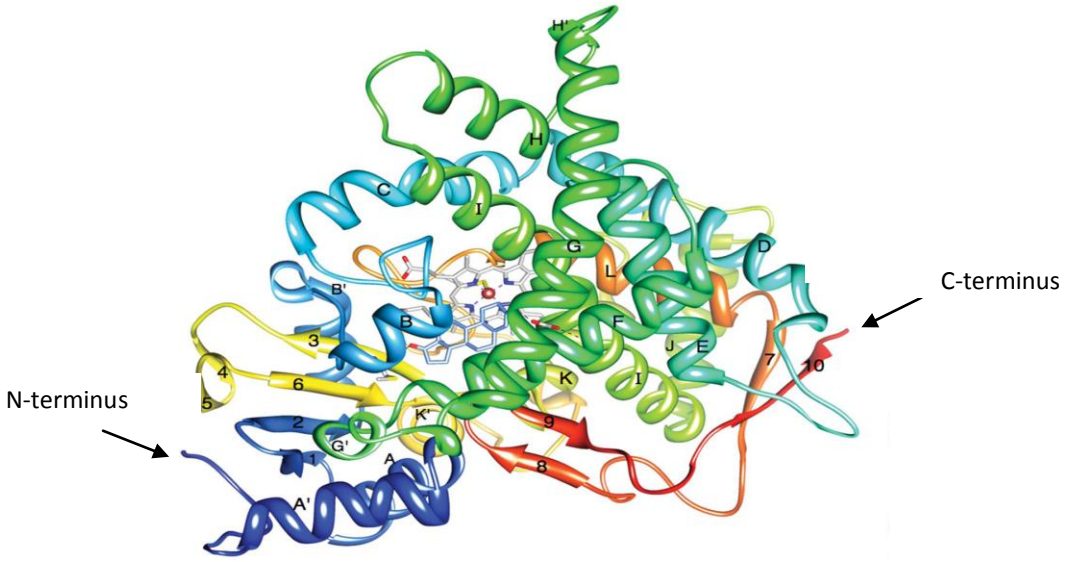


Figure 1.10: A ribbon structure of the human aromatase cytochrome 450. The N-terminus starting at residue 45 and the C-terminus starting at residue 496. The α-helices are labelled from A to L and β-strands are numbered from 1 to 10. Adapated from (Ghosh *et al.*, 2009).

In human, aromatase expression is regulated via the use of tissue-specific promoters. In endometriotic stromal cells and endometrial stromal cells from women with endometriosis, aromatase expression is regulated primarily by a region known as promoter II, which is the (Prostaglandin E_2) PGE_2/cAMP-responsive proximal promoter (Attar & Bulun, 2006a), as shown in *Figure 1.12*. The stimulatory transcription factor (SF-1) activates aromatase gene transcription in endometriosis by binding to aromatase promoter II. SF-1 is expressed specifically in endometriosis but not in eutopic endometrium. The region encoding the aromatase protein spans 30 kb and contains nine exons (II-X).

Although estrone is the major product of aromatase activity in endometriosis, it is however a weak estrogen and must be converted to E2 to exert a full estrogenic

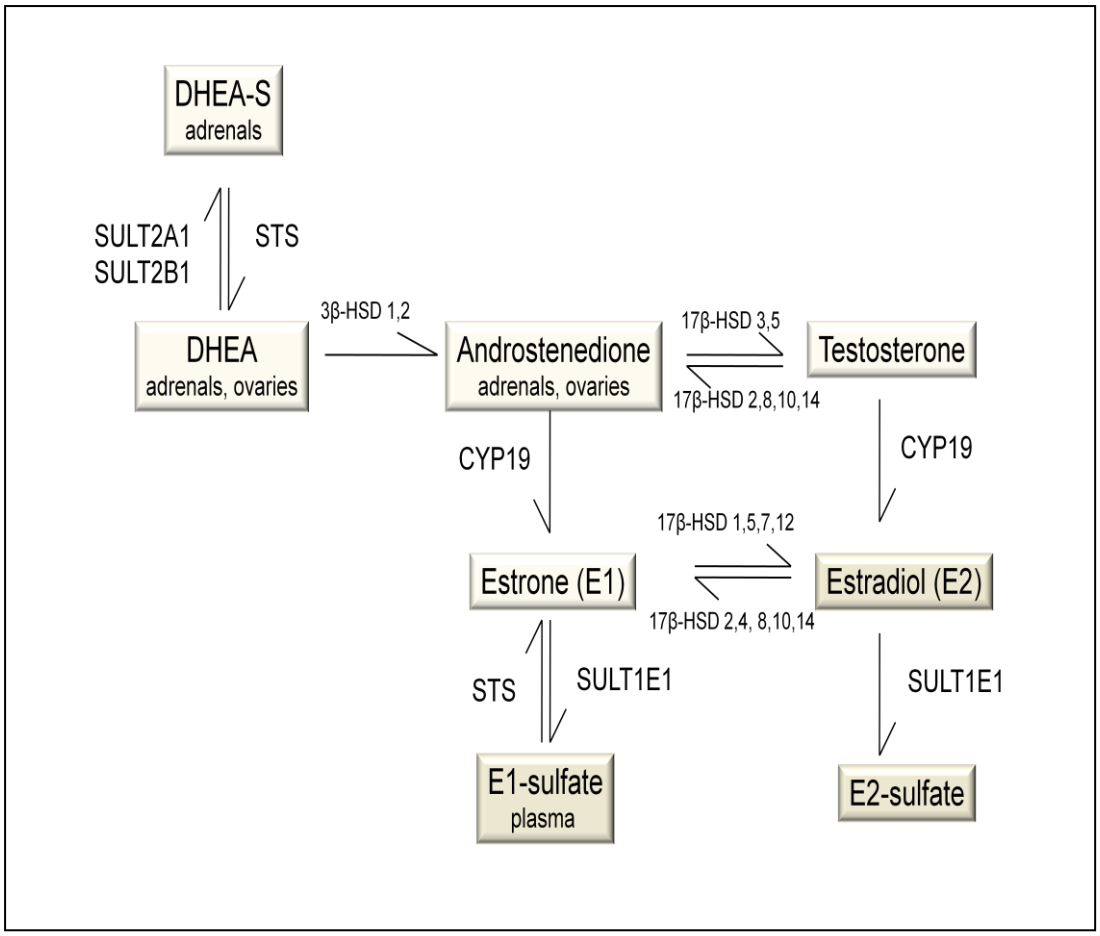


Figure 1.11: Estrogen biosynthesis and metabolism pathways. Estrogens can be produced from the inactive precursors of adrenal dehydroepiandrosterone sulfate (DHEA-S), dehydroepiandrosterone (DHEA) and androstenedione. Androstenedione is reduced to testosterone by 17β-HSD 3, 5. Androstenedione and testosterone are converted to estrone and estradiol respectively by CYP19 aromatase. In addition, the circulating estrone sulfate is converted to estrone by steroid sulfatase. Oxidative 17β-HSDs inactivate E2 and estrogen sulfotransferase inactivate both E1 and E2. E1 is also reduced to E2 by reductive 17β-HSDs. The above enzymes mentioned have been identified in both normal human endometrium and endometriotic cells. Adapted from (Rizner, 2009).

DHEA, dehydroepiandrosterone; E1, estrone; E2, estradiol; STS, steroid sulfatase; SULT1E1, estrogen sulfotransferase; SULT2A1, SULT2B1, DHEA sulfotransferases; 3β-HSD, 3β-hydroxysteroid dehydrogenase; 17β-HSD, 17β-hydroxysteroid dehydrogenases; CYP19, aromatase.

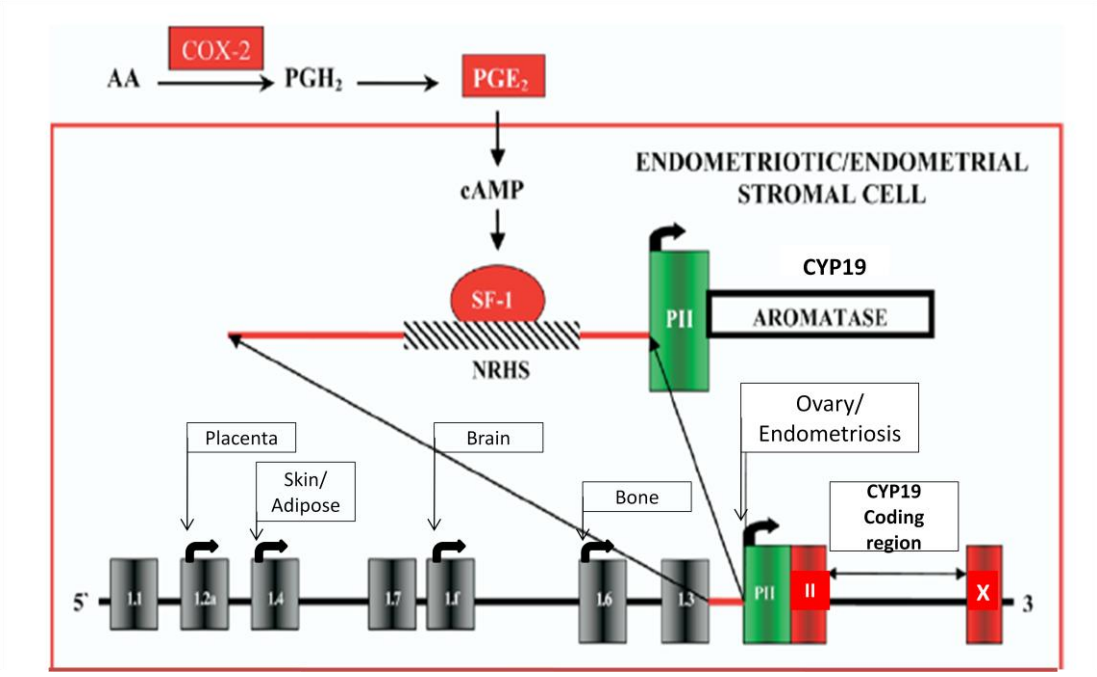


Figure 1.12: Regulation of aromatase (CYP19) gene expression by promoter II in endometriotic stromal and endometrial stromal cells. Cyclooxygenase-2 (COX-2) catalyzes the conversion of arachidonic acid to Prostaglandin E_2 (PGE_2). Both types of cell contain intact PGE_2 receptors that give rise to the formation of intracellular cyclic adenosine monophosphate (cAMP). cAMP induces binding of the transcription factor steroidogenic factor-1 (SF-1) to the proximal promoter II (PII) of the aromatase gene in endometriotic cells. The stimulatory transcription factor (SF-1) activates aromatase gene transcription. The promoters specific of different human tissues are shown above. Adapted from (Attar & Bulun, 2006b).

AA-arachidonic acid; COX-2, cyclooxygenase-2; NRHS-nuclear receptor half site.

effect (Bulun *et al.*, 2002b). Abundant aromatase expression and local estrogen production have been reported to occur in endometriotic stromal (Zeitoun & Bulun, 1999; Attar & Bulun, 2006a; Banu *et al.*, 2008) and endometriotic epithelial cells (Kitawaki *et al.*, 1997; Matsuzaki *et al.*, 2006). In contrast, in eutopic endometrium, aromatase expression was reported to be absent (Kitawaki *et al.*, 1997) or significantly low (Kyama *et al.*, 2008) when compared to endometriotic cells from patients with endometriosis.

Interestingly, others have compared the levels of aromatase in epithelial and stromal cells, and found a significant increase in aromatase mRNA in epithelial cells compared to stromal cells in both eutopic endometrium and endometriotic cells from patients with endometriosis (Matsuzaki *et al.*, 2006). Aromatase mRNA levels were significantly higher in ovarian endometriosis as compared to peritoneal endometriosis and deep endometriosis (Heilier *et al.*, 2006). The involvement of aromatase in endometriosis is further supported by a previous study showing that aromatase inhibitors can effectively alleviate chronic pelvic pain associated with endometriosis, which indicates that this inhibitor may suppress the progression of the disease (Patwardhan *et al.*, 2008).

Prostaglandins (PGs) particularly, Prostaglandin E_2 (PGE_2) are one of the active factors in peritoneal fluid (Attar & Bulun, 2006a). PGE_2 plays an essential role in the estrogen biosynthesis pathways in endometriotic cells as it is a potent inducer of aromatase and StAR (Noble *et al.*, 1997; Zeitoun *et al.*, 1999). It is produced and functions locally due to its instability and very short half life (Wu *et al.*, 2005). Peritoneal macrophages obtained from women with endometriosis have greater PG synthetic capability, compared with normal women (Wu *et al.*, 2002).

1.4.1.1.2 Cyclooxygenase-2

Cyclooxygenase (COX) is the rate-limiting enzyme in the metabolic conversion of arachidonic acid to PGs including PGE_2, a known mediator of inflammation and angiogenesis (Carli *et al.*, 2009). There are currently three known COX isoform: COX-1, COX-2 and COX-3, which are differently regulated (Scholz, 2003). Among these isoenzymes, COX-2 is the inducible form of COX that can be induced by several physiological and proinflammatory stimuli, including interleukin (IL)-1, tumor necrosis factor (TNF)-α and epidermal growth factor (EGF) (Scholz, 2003). As the biosynthesis of PGs is under the control of COX, it has a pivotal role in estrogen biosynthesis.

Estrogen up-regulates PGE_2 formation by stimulating COX-2 in endometriotic stromal cells (Zeitoun *et al.*, 1999). Inflammatory cytokines such as interleukin (IL)-6, IL-11 and tumour necrosis factor (TNF)-α promote local aromatase synthesis through COX-2 production, which maintain the estrogen supply in endometriotic tissue. Most recently, the levels of COX-2 mRNA and PGE_2 secretion were found significantly higher in normal endometrial stromal cells that were cultured with the peritoneal fluid from women with endometriosis compared to peritoneal fluid from normal women (Liu *et al.*, 2011). In contrast, COX-2 expression was found increased equally in both eutopic endometrium and endometriotic tissue of women with endometriosis (Wu *et al.*, 2005), which indicates that this enzyme is highly expressed in both types of cells.

1.4.1.1.3 17β-hydroxysteroid dehydrogenases

17β-hydroxysteroid dehydrogenases (HSDs) are enzymes that are responsible for reduction or oxidation of hormones, fatty acids and bile acids *in vivo* (Day *et al*.,

2008). The production of E2 can be also mediated through the 17β-HSDs. Multiples isoforms of 17β-HSDs have been characterised in human. Smuc & Rizner have divided 17β-HSDs according to the reaction they catalysed *in vivo*; reductive and oxidative enzymes. The reductive enzymes that catalyse the activation of estrone to E2 include 17β-HSD type 1, 5, 7 and 12 whereas the oxidative enzymes that catalyse the inactivation of E2 to estrone include 17β-HSD type 2, 4, 8, 10 and 14 (Smuc & Rizner, 2009), as shown in *Figure 1.11*.

Previous studies determined the expressions of 17β-HSD type 1, 7 and 12 were significantly higher in endometriotic cells compared to normal endometrium whereas no significant differences in the levels of 17β-HSD type 2, 4 and 8 were found between the two types of cells (Smuc *et al.*, 2009). In contrast, others demonstrated 17β-HSD type 2 was absent (Zeitoun *et al.*, 1998; Matsuzaki *et al.*, 2006) and low (Dassen *et al.*, 2007) in endometriotic cells as compared to normal endometrium, which impairs the inactivation of E2 to estrone that giving rise to increased local concentration of E2.

The 17β-HSD type 5 is also known as Aldo-keto reductase 1C3 (AKR1C3). To date, there are not many studies looking at the expression of AKR1C3 in endometriotic cells, however recently it has been determined that the mRNA expression of AKR1C3 was significantly increased in endometriotic cells as compared to without endometriosis (Smuc *et al.*, 2009). Besides acting as a 17β-HSD (type 5) that is involved in E2 synthesis, AKR1C3 is also one of the major human reductive 20α-hydroxysteroid dehydrogenases (20α-HSD) that catalyses the interconversion of active progesterone to the less active 20α-hydroxyprogesterone (20α-OHP) (Rizner *et al.*, 2006) that have lower affinities towards the progesterone receptors (Pollow *et al.*, 1975).

1.4.1.1.4 Sulfatase and sulfotransferase

The sulfatase pathway can also synthesise E2 (Purohit *et al.*, 2008; Smuc & Rizner, 2009). In this pathway, the circulating steroid sulfatase (STS) converts estrone-sulfate to estrone while estrone sulfotransferase (SULT1E1) inactivates the estrone, forming their sulfates (Rizner, 2009; Smuc & Rizner, 2009), as shown in *Figure 1.11*. Previous studies have determined high concentrations of estrone-sulfate in blood and tissues, which indicates this enzyme as a reservoir for the formation of active estrogens via the action of STS (Noel *et al.*, 1981). Previous studies have detected a significant increase of STS and no significant differences in SULT1E1 between endometriotic cells and normal endometrium (Smuc *et al.*, 2009). The similar role of 17β-HSDs in the aromatase pathway is occuring here to form E2. Therefore, the expression of estrone is also depends on the amount of STS and SULT1E1. However, there is limited published data on this alternative pathways in endometriosis.

1.4.1.2 Estrogen receptors function and distribution

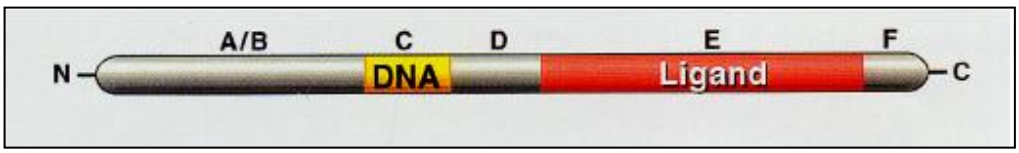
The capacity of different tissue to respond to steroids is determined according to the concentrations of the relevant receptors (Bergqvist *et al.*, 1997). The influence of estrogen on target organs is modulated by the local distribution of estrogen receptors (ERs); ER-alpha (α), ER-beta (β) and a G-protein coupled receptor (GPR)30 (Hasbi *et al.*, 2005). ER-α and ER-β belong to the nuclear receptor superfamily, a family of ligand-regulated transcription factors, (Moggs & Orphanides, 2001; Pettersson & Gustafsson, 2001; Rizner, 2009) whereas GPR30 is a membrane estrogen receptor (mER) (Hasbi *et al.*, 2005). However, in the study of endometriosis, only ER-α and ER-β have been determined in endometrium and endometriotic cells (Brandenberger *et al.*, 1999; Hudelist *et al.*, 2005; Xue *et al.*, 2007; Rizner, 2009; Bulun *et al.*, 2010). The structure of the nuclear receptor

superfamily is shown in *Figure 1.13.* GPR30 is widely distributed in different tissues including heart, breast, lung, vascular endothelium and leukocytes (Hasbi *et al.*, 2005). This receptor has been shown to be highly expressed in primary H-38 endometrial cancer cells and in Ishikawa endometrial cancer cell line that are responsible for promoting cancer cell survival and proliferation via MAPK and PI3K signal transduction cascades (Lin *et al.*, 2009). However, it has not yet been implicated in endometriosis.

ER-α and ER-β have been identified as having different tissue distribution, binding affinity, and biological function. ER-α is highly expressed in classical estrogen target tissues such as the uterus, placenta, pituitary and cardiovascular system, whereas ER-β is more abundant in the ventral prostate, urogenital tract, ovarian follicles, lung, and immune system (Roman-Blas *et al.*, 2009). In the normal human endometrium, both ERs are expressed in both epithelial and stromal cells (Lecce *et al.*, 2001). The ER-α and ER-β proteins are about 47% identical and encoded by distinct genes located on chromosome 6 (q24-q27) and 14 (q21-q22), respectively (Roman-Blas *et al.*, 2009). Comparing to ER-α, ER-β is shorter by about 80 amino acids. The structures of both receptors are shown in *Figure 1.13.*

In the endometrium of normal women and patients with endometriosis both ER-α and ER-β have been detected with the mRNA expression of ER-α being significantly higher than ER-β (Matsuzaki *et al.*, 2000; Bulun *et al.*, 2010). Both receptors are also present in endometriotic cells (Hudelist *et al.*, 2005); however, a number of researchers have demonstrated elevated levels of ER-β mRNA expression over ER-α when compared to normal endometrium (Brandenberger *et al.*, 1999; Hudelist *et al.*, 2005; Xue *et al.*, 2007). In addition, by using immunocytochemical analyses,

a)



b)

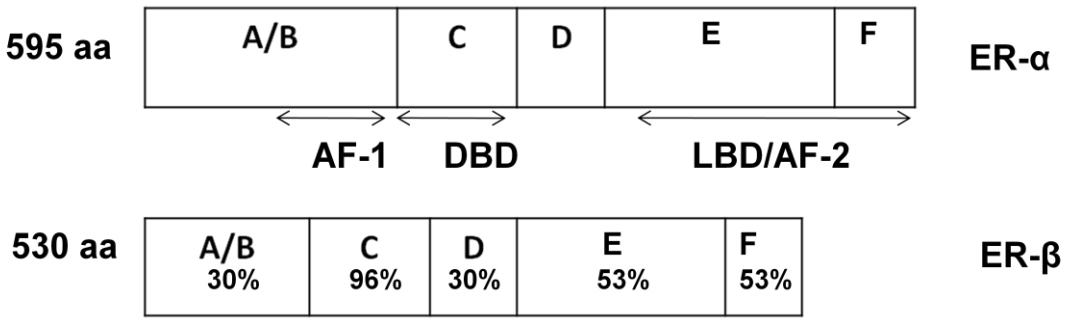


Figure 1.13: Structures of a typical of nuclear receptor, ER-α and ER-β.

a) The nuclear receptors share common structure and functions domain. A typical nuclear receptor consists of; a variable N-terminal region that contributes to transcriptional activation (A/B), a conserved DNA-binding domain (DBD), which targets the receptor to specific DNA sequences known as hormone response element (C), a variable hinge region, a less characterized region (D), a conserved ligand-binding domains (LBD) that harbors the ligand binding pocket (E) and a variable C-terminal region (F). Adapted from (Mangelsdorf *et al.*, 1995)

b) Structures of ER-α and ER-β, which are almost identical in the DBD region. ER contains two autonomous transcriptional activation functions domains: the AF-1 domain, located in the N terminus, and AF-2, located within the LBD. The percent of homology is indicated in the diagram. Adapted from (Roman-Blas *et al.*, 2009).

ER-β was found to be the dominant receptor in endometriotic cells as compared to ER-α or progesterone receptor (Fazleabas *et al.*, 2002). Suppression of ER-α mRNA and proteins by ER-β is one of the possible reasons to explain the difference in their expression (Hastings & Fazleabas, 2006). These authors showed that ER-β knockdown significantly increased ER-α mRNA and protein levels in endometriotic stromal cells.

In an animal study using baboon where endometriosis was induced by inoculation of endometrial currettings into the peritoneal cavity, ER-α was found in both eutopic and ectopic endometrium whereas ER-β was only expressed in the ectopic endometrium (Fazleabas *et al.*, 2003). The specific induction of ER-β suggests this receptor may play a critical role in the establishment and maintenance of the disease.

Conversely, others have found a significant increase in ER-α compared to ER-β in both eutopic endometrium and endometriotic cells from patients with endometriosis, leading to the proposal that the principal and regulatory effects of E2 are mediated mainly via ER-α rather than ER-β (Matsuzaki *et al.*, 2001). In another animal study using mice, induction of endometriosis by injecting a mixed population of human immortalized endometriotic epithelial and stromal cells have demonstrated an abundant expression of ER-α, but not ER-β in both endometriotic epithelial and stromal cells (Banu *et al.*, 2009).

Despite extensive investigations into the role of ERs in endometriosis, most of the results were inconclusive and the biological roles of both receptors, however, remain obscure. Furthermore, the local regulation and expression of ERs in endometriotic cells is poorly understood despite the expression patterns of ERs in endometriotic lesions have been reported to be different from those in the eutopic endometrium.

1.4.1.3 Mechanism of estrogen action in endometrium and endometriotic cells

The mechanism of estrogen action in normal endometrium and endometriotic cells, can be described as falling into two categories; i. Estrogen Response Element (ERE)-dependent pathway and ii. Non Estrogen Response Element (ERE)-dependent pathway.

With regards to the ERs, Moggs & Orphanides have proposed that tissue responsiveness to estrogens is determined by cell-type specific expression pattern of ER subtypes and their coregulators (Moggs & Orphanides, 2001). The ERs modulate the transcription of target genes when bound to ligand (Pearce & Jordan, 2004). Thus, the distinct functions of ER-α and ER-β are dependant on the nature of bound ligands, post-translational modifications, cofactor interactions and promoter response elements, as shown in *Figure 1.14*.

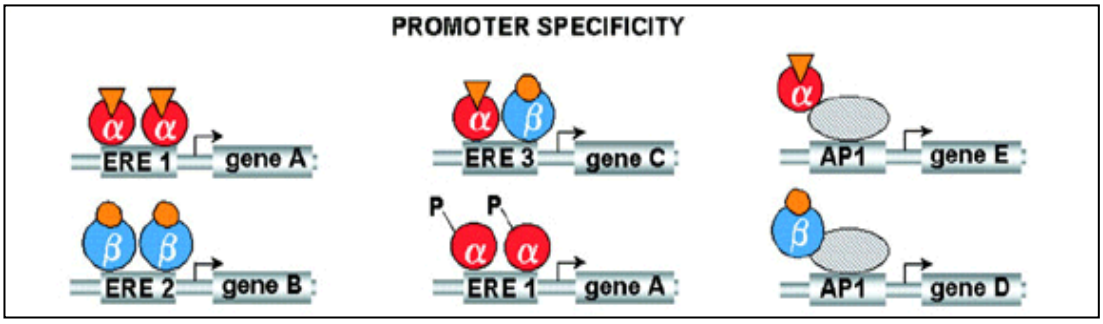


Figure 1.14: Promoter specificity of Estrogen Receptors subtypes. ERs can bind directly to the promoters of estrogen-responsive genes through estrogen response elements (ERE) as homo- or heterodimers, or indirectly through DNA-bound transcription factors such as *c-fos* and *c-jun* at activating protein-1 (AP-1) binding sites. Protein kinase cascades are involved in the ligand-independent association of ERs. Adapted from (Moggs & Orphanides, 2001).

1.4.1.3.1 Estrogen Response Element (ERE)-Dependent pathway

There is compelling evidence showing that E2 exerts most of its effects by binding

to its intracellular receptors (ER-α and ER-β), which then go on to interact with the Estrogen Response Element (ERE) in regulated promoters (Guzeloglu Kayisli *et al.*, 2004; Seval *et al.*, 2006). Once bound by estrogens, conformational changes in the ERs are triggered and ER homo- or hetero-dimerization occurs (Hasbi *et al.*, 2005), as shown in *Figure 1.14*. The E2-receptor complex translocates to the nucleus and acts as a transcription factor that binds to ERE, a specific regulatory DNA sequence, in the promoter regions of their target genes, and induces gene transcription (Hasbi *et al.*, 2005). The resulting enhancement of gene expression leading to an increase in synthesis of target genes products. This estrogen pathway that involves ERE is also known as the *'genomic' or 'classical'* pathway (Belcher & Zsarnovszky, 2001; Guzeloglu Kayisli *et al.*, 2004; Seval *et al.*, 2006). The promoters of genes encoding human oxytocin, prolactin, cathepsin D, progesterone receptor, vascular endothelial growth factor, insulin-like growth factor (IGF)-1, or c-fos genes are amongst those that contain an ERE and are activated by this pathway (for reviews see (Klinge, 2001). The mechanism of ERE-dependent induction is not instant and takes several hours before producing its effects. Both ER-α and ER-β have been shown to mediate its action via this pathway in endometrial and endometriotic cells (Bulun *et al.*, 2010), as shown in *Figure 1.15* as pathway 1.

1.4.1.3.2 Non Estrogen Response Element (ERE)-Dependent pathway

In addition to the involvement of the ERE, the ligand-bound ERs (both ER-α and ER-β) can also regulate genes that lack an ERE, via protein-protein interactions with other transcription factors at specificity protein 1 (Sp1) and AP-1 binding sites in endometriotic cells (Bulun *et al.*, 2010), as shown in *Figure 1.15* as pathway 2. Another proposed mechanism of estrogen action that has been demonstrated previously in endometriotic and endometrial cells involves intracellular signal transduction cascades, PI3K/Akt and MAPKs including ERK1/2 and p38 MAPK. Although previous studies have demonstrated E2 induced the phosphorylation of

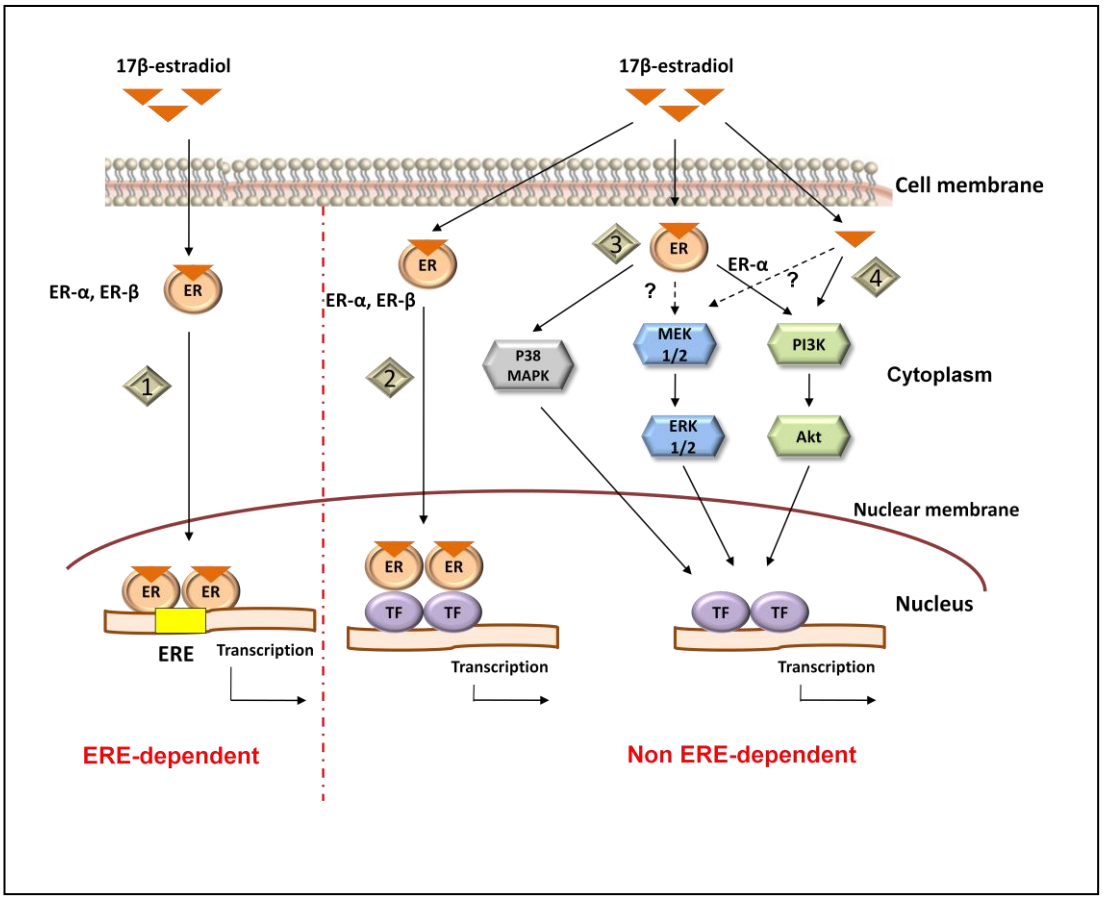


Figure 1.15: The regulation of estrogen action in endometrium and endometriotic cells.

ERE-dependent pathway (Pathway 1): Ligand-activated ERs bind specifically to EREs in the promoter of target genes leading to the induction of gene transcription (Guzeloglu Kayisli *et al.*, 2004; Seval *et al.*, 2006; Bulun *et al.*, 2010). **Non ERE-dependent pathway; Pathway 2**: Ligand-bound ERs interact with transcription factors, whose promoters do not have or lack of EREs (Bulun *et al.*, 2010). **Pathway 3**: Ligand-bound ERs activate the p38 MAPK (Seval *et al.*, 2006) and PI3K/Akt signal transduction pathway (Wilhelm *et al.*, 2011). **Pathway 4**: E2 directly activates PI3K/Akt pathway without ERs (Guzeloglu Kayisli *et al.*, 2004).
TF, transcription factor.

PI3K/Akt (Zhang *et al.*, 2010) and MAPK/ERK1/2 (Murk *et al.*, 2008; Zhang *et al.*, 2010) in primary endometriotic cells, the exact mechanism has not been clarified. However, the activation of PI3K/Akt signaling pathway by ligand-activated ER-α was demonstrated in the endometrium of post menopausal women (Wilhelm *et al.*, 2011) as shown in *Figure 1.15* as pathway 3, and in endothelial cells (Simoncini *et al.*, 2003) whereas in the brain, the activation of both PI3K/Akt and MAPK/ERK1/2 signaling pathways by ligand-activated ER-α and ER-β has been demonstrated (Belcher & Zsarnovszky, 2001). This may explain one of the possible mechanisms of cell proliferation in postmenopausal women. The activation via PI3K/Akt and MAPK occurs rapidly, within seconds to a few minutes (Belcher & Zsarnovszky, 2001; Guzeloglu Kayisli *et al.*, 2004; Seval *et al.*, 2006; Zhang *et al.*, 2010). E2 has also been shown to activate the p38 MAPK *in vivo* and *in vitro* (Seval *et al.*, 2006). These authors have demonstrated *in vivo* p38 MAPK phosphorylation in the endometrium by immunohistochemistry and *in vitro* in endometrial stromal cells and epithelial cells by Western blot analysis and ELISA. In addition, the p38 MAPK phosphorylation in endometrial stromal cells was inhibited by a specific p38 MAPK inhibitor, SB203580 and ER antagonist ICI 182780 inhibited p38 phophorylation in both endometrial stromal cells and endometrial epithelial cells, suggesting the involvement of the ER; however the type of ER was not investigated.

Interestingly, in contrast to other non ERE-dependent pathways, E2 has also been demonstrated to directly activate PI3K/Akt signaling pathway *in vitro* without the involvement of ER (Guzeloglu Kayisli *et al.*, 2004) as shown in *Figure 1*.15 as pathway 4. Normal human endometrial cells were used in that study to determine the Akt phosphorylation by E2. The results from the three different techniques; Western blotting, immunohistochemistry and immunocytochemistry revealed that E2 significantly increased Akt phosphorylation, which was inhibited by PI3K inhibitior, Wortmannin. However, the used of an ER antagonist, ICI 182780 did not inhibit Akt phosphorylation. This inhibitor produces conformational changes in the ER that prevent it from binding to ERE. Therefore, it is proposed that the role of E2

in cell proliferation is also occurring via a non-ER and non-ERE-dependent manner through the PI3K/Akt pathway.

Although estrogen is well known to stimulate cell proliferation by activating target genes that promote cell cycle progression, the target genes that are involved in the above studies of endometriosis have not been well clarified. Previously, ER-responsive genes including cyclin D1 and *c-myc* have been implicated in breast cancer disease (Foster *et al.*, 2001). Cyclin D1 is an important regulator of G1 to S-phase transition in cell cycle (Alao *et al*., 2006) whereas the c-myc gene produces an oncogenic transcription factor involved in cell growth, cell proliferation, apoptosis and cellular metabolism (Boxer & Dang, 2001). Unfortunately, the role of both of the genes in endometriosis is poorly understood although previous studies have determined cyclin D1 mRNA was abundantly expressed in human endometrial and endometriotic cells (Banu *et al.*, 2008). A suppression of cyclin D1 gene expression in human endometrial epithelial cells by Dienogest, a synthetic progestin however support the role of cyclin D1 in endometriosis (Shimizu *et al.*, 2009). Although both endometriotic cells and cancer cells proliferate in order to survive, the endometriotic cell growth is in control fashion and normally it directly invade the local region as this disease is considered as a benign disease (Ngo *et al*., 2009) whereas the cancer cells grow uncontrollably and often metastasize. Thus, it is possible that both of the genes have different effects or regulation on endometriotic cells and cancer cells.

1.4.2 Roles of reactive oxygen species in endometriosis

Reactive oxygen species (ROS) are a collection of chemically-reactive molecules that include both oxygen radicals and certain non radicals that are oxidising agents and/or are easily converted into radicals (Halliwell, 2006). Examples of free radical ROS include the superoxide anion ($O_2^{\cdot-}$), and the hydroxyl ($OH^{\cdot}$), hydroperoxyl ($HO_2^{\cdot}$)

and carbonate ($CO_3^{\cdot}$) radicals, whereas examples of nonradical ROS are hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), ozone (O_3) and peroxynitrite ($ONOO^-$). ROS are by-products of numerous metabolic pathways, but mitochondria are the major cellular site where O_2 is reduced and superoxide anion is released. Thus, ROS production is directly proportional to cell activity (Hernandez-Garcia *et al.*, 2010). ROS are also more generally known as oxidants. The effects of ROS on cell activity and function depends on the cell type and the levels of ROS applied (Halliwell, 2006). The mechanisms of cellular response to ROS is shown in *Figure 1.16*.

1.4.2.1 Oxidative damage by elevated ROS

Production of ROS is approximately balanced with antioxidant defence systems in healthy aerobes (Halliwell, 2007). Cells upregulate their defence and/or repair systems to cope with the presence of increased ROS, for example by increasing the levels of protective enzymes including heme oxygenase-1 (HO-1), superoxide dismutase (SOD) as well as non-enzymatic defenses such as glutathione (GSH). A disturbance in the prooxidant-antioxidant balance leads to oxidative damage where there is biomolecular damage to the constituent molecules of living organisms (Conde de la Rosa *et al.*, 2006). Thus, oxidative damage can result not only from increased ROS, but also decreases in enzymatic and non-enzymatic antioxidant levels (Valko *et al.*, 2007). The biomolecular damage due to high concentrations of ROS involves damage to nucleic acids, lipids and proteins (Valko *et al.*, 2007).

1.4.2.1.1 Oxidative damage to DNA

Elevated ROS in cells and tissues usually refers to increased generation of superoxide anion ($O_2^{\cdot-}$) and H_2O_2 (Halliwell, 1987). However, neither $O_2^{\cdot-}$ nor H_2O_2 are very reactive unless there is a participation of the transitional metal ions iron or copper that convert poorly reactive species into more reactive one. It has been reported that H_2O_2 exposure leads to the formation of a highly reactive species, the

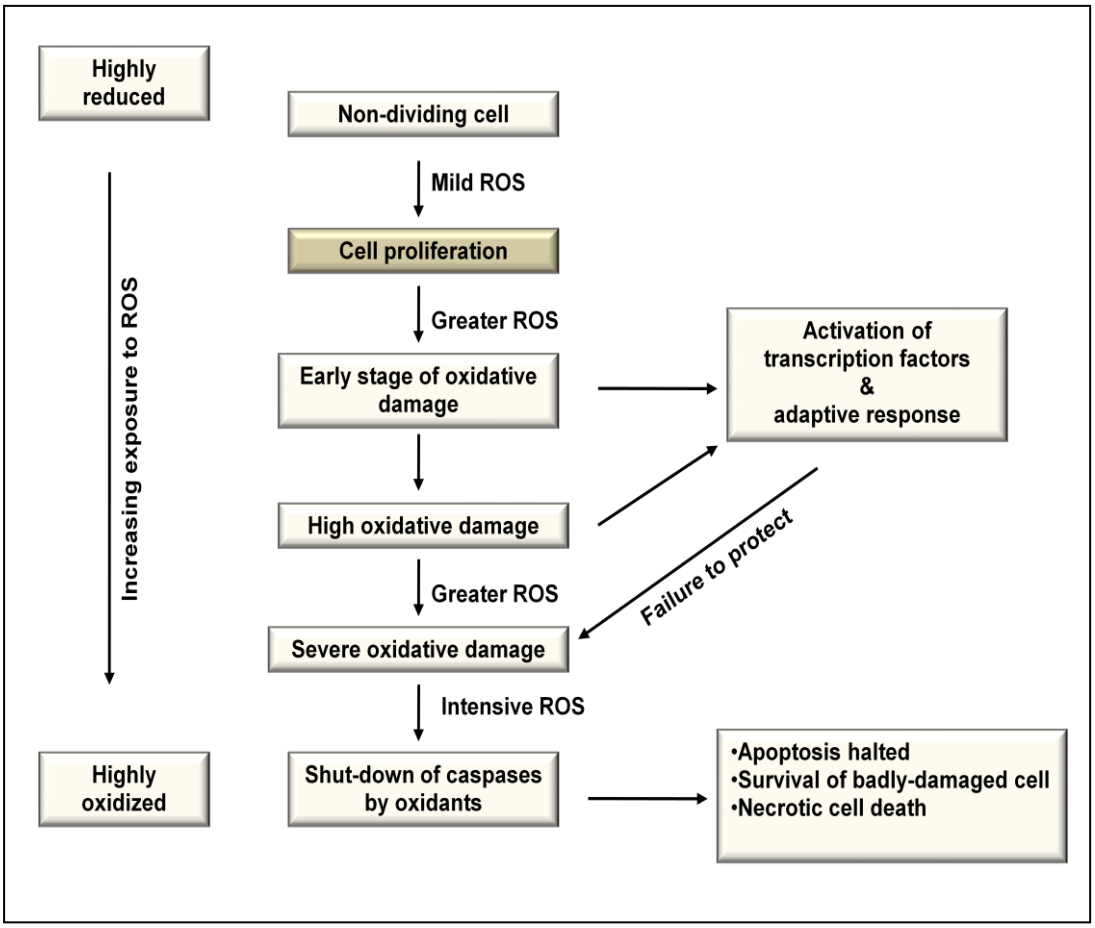


Figure 1.16: Cellular response to different levels of ROS. Mild ROS can cause cell proliferation. Greater ROS usually halts the cell cycle, or can drive cells into senescence where the cell survives without no longer divide. Severe oxidative damage however, trigger cell death by apoptosis, necrosis or both. Adapted from (Halliwell, 2007).

hydroxyl radical ($OH^{\cdot}$) in the presence of these transition ions (Halliwell, 1987). The hydroxyl radical is known to react with many parts of the DNA molecule, damaging the purine and pyrimidine bases and the deoxyribose backbone (Marnett, 2000).

1.4.2.1.2 Oxidative damage to lipids

Previous studies have reported that metal-induced generation of ROS also attacks cellular components including the polysaturated fatty acid (PUFAs) residues of phospholipids leading to generation of highly cytotoxic aldehydes (Halliwell, 1987). The breakdown of PUFAs results in the formation of lipid peroxidation (LPO) products such as reactive aldehydes malondialdehyde (MDA), 4-hydroxy-2-nonenal (HNE) and acrolein (Rahman & Adcock, 2006; Nair *et al*., 2007; Valko *et al*., 2007). These products are capable of causing massive DNA damage and deregulation of cell homeostasis (Nair *et al.*, 2007). *Figure 1.17* shows proposed formation of acrolein (C_3H_4O) from arachidonic acid, a polyunsaturated omega-6 fatty acid.

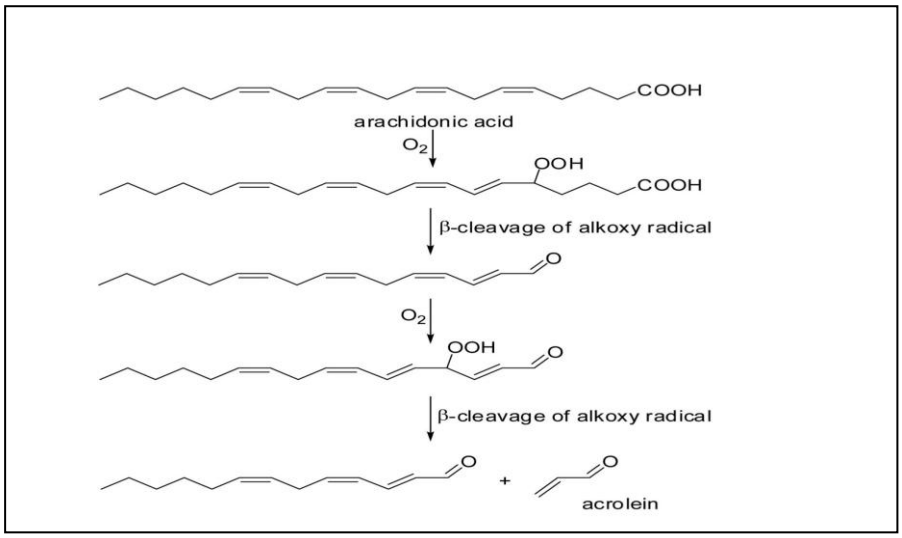


Figure 1.17: Mechanism of acrolein formation from lipid peroxidation of polyunsaturated fatty acid (PUFAs). There are two main reactions involved that result in carbon-carbon bond cleavage of lipid hydroperoxides, i.e β-cleavage of the corresponding alkoxy radical. Adapted from (Esterbauer *et al*., 1991).

1.4.2.1.3 Oxidative damage to proteins

The importance of protein oxidation by ROS has been proved previously where amino acids and simple peptides that were exposed to ionising radiation which resulted in the formation of hydroxyl radicals or a mixture of hydroxyl/superoxide radicals which could damage side chains of all amino acids, in particular cysteine and methionine (Stadtman, 2004). Oxidative damage to proteins may seriously affect function, for example of receptors, enzymes and transport protein (Halliwell & Whiteman, 2004).

1.4.2.2 Cell proliferation by sub-lethal ROS

Although high intracellular levels of ROS have the potential to cause major cellular damage, it is interesting to note that cells respond to lower levels of ROS by proliferating, as shown in *Figure 1.16*. As ROS have the potential to induce cell proliferation, their roles in the mechanism of endometriotic cell proliferation have been studied by several researchers. Levels of ROS were found significantly higher in peritoneal fluid (Wang *et al.*, 1997) and serum (Shanti *et al*., 1999) from women with endometriosis as compared to normal women. In addition, previous *in vitro* studies have demonstrated ROS at low concentrations induced cell proliferation in endometrium from women with endometriosis (Foyouzi *et al.*, 2004; Wu & Guo, 2006). Roles of ROS in endometriosis will be described further in subsequent chapters. Although ROS have been implicated in the pathogenesis of endometriosis, the molecular basis of ROS induce cell proliferation in endometriosis remains far from clear. An antioxidant is a molecule that capable of inhibiting the effects of ROS, thus it has been implicated in the pathogenesis of endometriosis. Roles of antioxidants are described further in Chapter 6 and Chapter 7.

1.5 Roles of signaling pathways in endometriosis

Cell signaling or signal transduction describes cell-cell communication and their response to extracellular stimuli through biological mechanisms. Signal transduction that is triggered by extracellular signals such as hormones, growth factors, cytokines and neurotransmitters enables information to be transmitted from the extracellular to various functional elements intracellularly (Thannickal & Fanburg, 2000). Transcription factors transmit signals to the cell nucleus leading to the expression of certain genes (Valko *et al.*, 2007). Numerous studies have been conducted to identify the signaling pathways that are involved in the mechanism of endometriotic cell proliferation including MAPK pathways and PI3K signaling pathways.

1.5.1 Roles of MAPK signaling pathways in endometriosis

MAPK pathways are among the pathways that use protein phosphorylation to transduce signals from the plasma membrane to the nucleus and other cellular targets (Strniskova *et al.*, 2002). There are three major MAPK pathways have been identified in mammals that have been named according to the terminal MAPK proteins; i) the extracellular signal-regulated protein kinase (ERK) cascade ii) p38-MAPK cascade and iii) c-Jun NH_2-terminal protein kinase (JNK) cascade (Krauss, 2003). Although each MAPK has unique characteristics, they share some of the features where each protein kinase cascade consists of at least three enzymes that are activated in series : a MAPK Kinase Kinase (MAPKKK or MAP3K or MEKK), a MAPK Kinase (MAPKK or MEK or MKK) and a MAP kinase (MAPK) (Zhang & Liu, 2002). Cells will exert their biological cell responses via these pathways including cell proliferation, differentiation, development, transformation and apoptosis, as shown in *Figure 1.18* (Zhang & Liu, 2002).

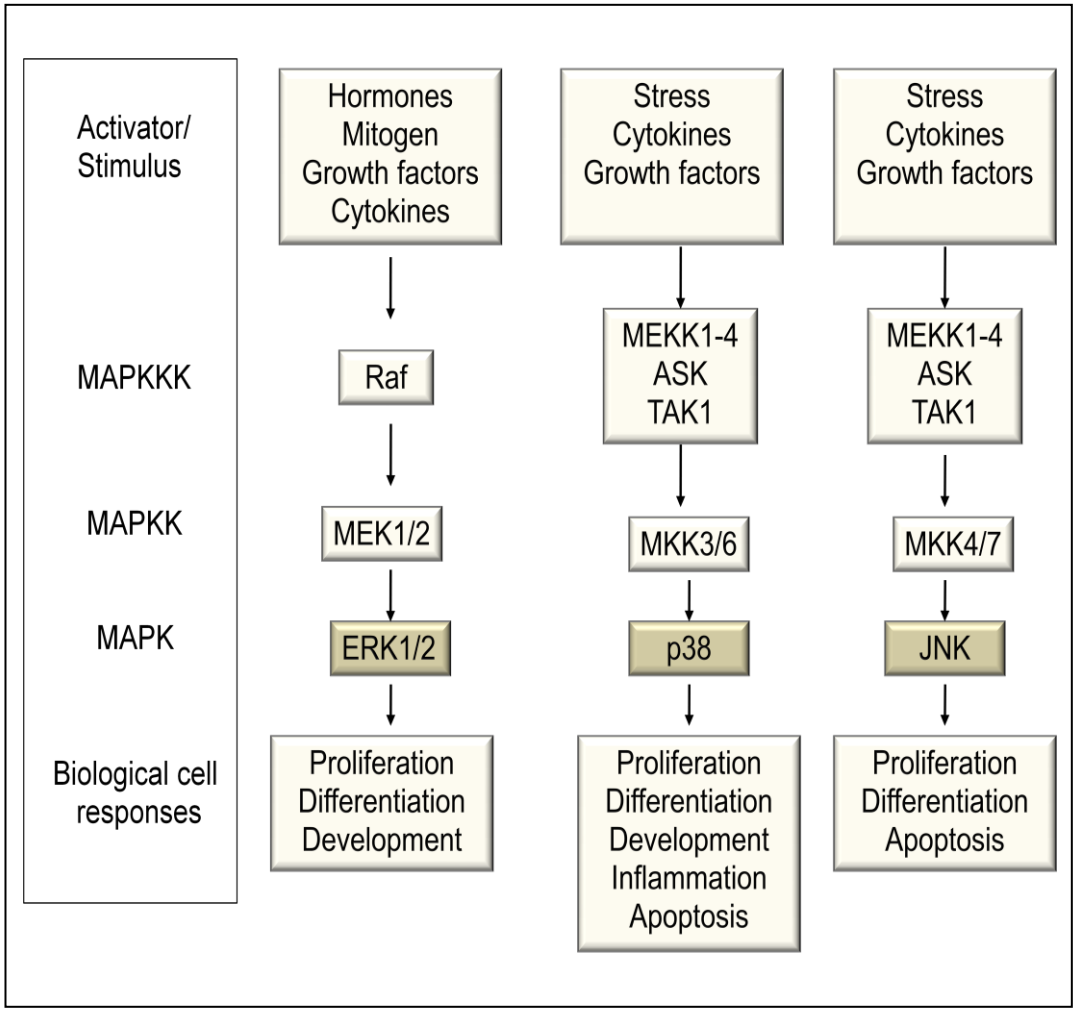


Figure 1.18: Major MAPK pathways in mammals. The pathways including ERK1/2, p38 MAPK and JNK. Adapted from (Zhang & Liu, 2002).

The ERK pathway is activated by various factors such as growth factors, cytokines and osmotic stress, and has been implicated as a key regulator of cell proliferation and prevention of apoptosis (McCubrey *et al.*, 2006). There is a growing body of evidence implicating this pathway to be induced by ROS in many cell types such as pulmonary epithelial cells (Buder-Hoffmann *et al.*, 2001) and vascular smooth muscle cells (Blanc *et al.*, 2003).

The p38 MAPK has the p38 protein as a characteristic terminal kinase. There are four known p38 isoforms including α, β, γ and δ, which are strongly activated by environmental stresses, inflammatory cytokines, hyperosmotic shock, metabolic stress and ultraviolet light (Strniskova *et al.*, 2002). It is generally accepted that p38 activity is mainly involved in normal immune and inflammatory response, therefore p38 is mainly activated in macrophages, neutrophils and T-cells by various extracellular mediators of inflammation (Roux & Blenis, 2004).

JNK is a stress-activated protein kinase that can be induced by inflammatory cytokines, bacterial endotoxin, osmotic shock, UV radiation and hypoxia (Bennett *et al.*, 2001). These authors reported that three JNK genes (JNK1, -2, -3) have been identified in humans with JNK1 and JNK2 have a broad tissue distribution.

A group of investigators has determined 79 pathways with more than 100 genes in endometriotic epithelial tissue from 12 women with endometriosis using cDNA microarray technology, which provides a powerful tool for quantifying expression levels of thousands of genes simultaneously (Wu *et al.*, 2006). These authors have identified 13 genes that are involved in MAPK pathways including MAPK6, MAPK7 and RAF1. Differences in gene expression between normal endometrium and endometriotic cells have also been studied previously using Serial Analysis of Gene Expression (SAGE) (Honda *et al.*, 2008). SAGE is another gene expression profiling technique that is a sequencing-based whereas microarray technology is

hybridization-based. Among the genes, AXL receptor tyrosine kinase (AXL) and SHC (Src homology 2 domain containing)1 were found to be up-regulated in endometriotic cells and this was further validated by immunohistochemical studies, that showed increased expression of both genes in both epithelial and stromal cells of the endometriotic tissues compared to normal endometrial tissues.

The involvement of the three major MAPK pathways using endometriotic stromal cells has been studied previously (Yoshino *et al.*, 2004). This has revealed that the phosphorylation of ERK1/2, p38 MAPK and JNK/SAPK was stimulated by IL-1β, TNF-α and H_2O_2. In that study, the effects of specific inhibitors for each MAPK, PD98059 (ERK1/2 MAPK), SB202190 (p38 MAPK) and SP600125 (JNK/SAPK) on IL-1β secretion of pro-inflammatory cytokines (IL-6 and IL-8) and COX-2 expression in endometriotic stromal cells were also examined. Both SB202190 and SP600125 suppressed IL-1β-induced secretion of IL-6 and IL-8 whereas PD98059 was only suppressed IL-1β-induced secretion of IL-8. In addition, both SB202190 and PD98059 suppressed IL-1β-induced expression of COX-2. Therefore, it can be concluded that ERK1/2, p38 MAPK and JNK/SAPK are present in endometriotic cells and are activated by pro-inflammatory cytokines as determined by Western blot analysis. Thus MAPK has a pivotal role in the mechanism of cell proliferation in endometriosis.

The induction of ERK1/2 activity by E2 and progesterone was determined in endometrial stromal cells using Western blotting (Murk *et al.*, 2008). In that study, endometrial stromal cells were cultured with E2 at 10^{-8} M or progesterone at 10^{-7} M only, or with the combination of both E2 at 10^{-8} M and progesterone at 10^{-7} M. The results revealed the combination of both E2 and progesterone increased ERK1/2 activity significantly as compared to untreated endometrial stromal cells. However, E2 or progesterone alone did not induce ERK1/2 phosphorylation. Thus, they proposed that cells that are not normally responsive to estrogen are instead activated via ERK1/2 signaling, which may explain the ability of cells to continue grow and proliferate in the peritoneum after retrograde menstruation. In contrast

to endometrial stromal cells, these authors demonstrated E2 at 10^{-8} M alone was able to stimulate ERK1/2 phosphorylation in endometrium of women with endometriosis, which further support the important role of this pathway in the pathogenesis of endometriosis. Recently, an *in vitro* study using primary endometriotic epithelial cells has also shown that E2-induced endometriotic epithelial cell proliferation involved MAPK/ERK 1/2 pathway (Zhang *et al.*, 2010).

1.5.2 Roles of phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathways in endometriosis

Inositol-containing phospholipids of the plasma membrane are the beginning compounds for the formation of various inositol messengers in response to various intra- and extracellular signals (Krauss, 2003). Phosphatidylinositol-3 kinases (PI3Ks) are characterised by their ability to convert phosphatidylinositol-4,5 phosphate (PIP_2) into phosphatidylinositol-3,4,5 phosphate (PIP_3) (McCubrey *et al.*, 2006). As shown in *Figure 1.19*, PI3K is activated via three pathways: i) Direct interaction with activated receptor tyrosine kinase ii) Activation via the Ras protein and iii) Activation by the βγ-subunits of G protein that liberated upon activation of G protein-coupled receptors, GPCR (Krauss, 2003). Approximately more than 16 different classes of receptor tyrosine kinases have been identified including Epidermal Growth Factor (EGF) receptor family, Insulin receptor family, Platelet-derived Growth factor (PDGF) receptor family and Fibroblast Growth Factor (FGF) receptor family. The conversion of PIP_2 to PIP_3, which functions as a membrane-localized messenger results in membrane localisation of phosphoinositol-dependent kinase-1 (PDK1) and the serine/threonine protein kinase B (PKB) and their pleckstrin homology (PH) domain.

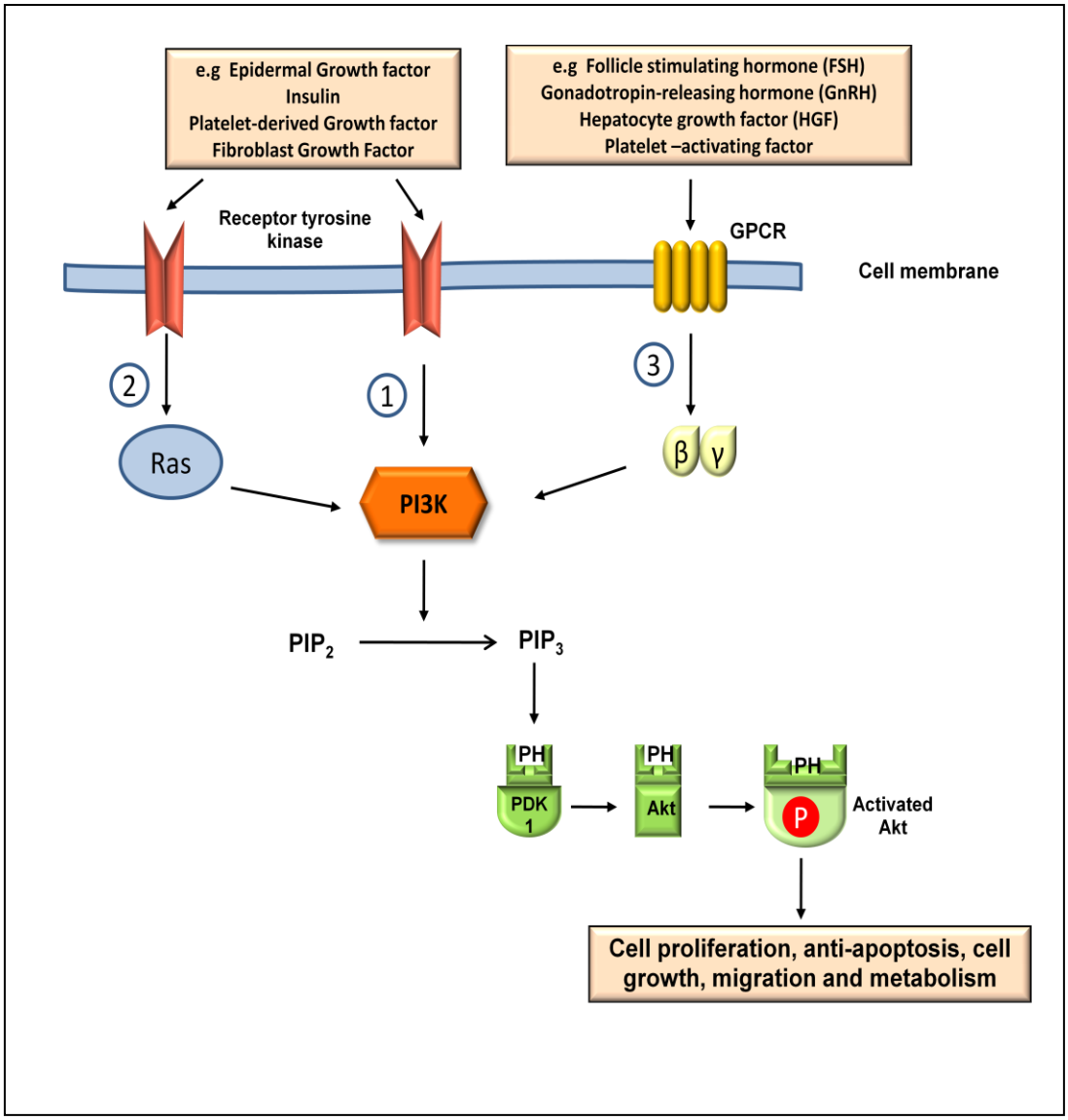


Figure 1.19: Pathways of PI3K activation. PI3K can be activated by growth factor receptors either 1. Direct interaction 2. via the Ras protein or 3. via the activation of βγ-subunits of G protein. PI3K converts PIP_2 to PIP_3 leading to membrane localisation of PDK1 and Akt. Activated Akt stimulates various reactions including cell proliferation, inhibit apoptosis, cell growth, cell migration and metabolism of glucose and lipids. Adapted from (Krauss, 2003).

Full activation of PKB (also known as Akt kinase) requires phosphorylation by PDK1. Subsequently, activated Akt phosphorylates a variety of target proteins that involved in cell survival, growth, migration, proliferation, metabolism (lipid and glucose) (Liao & Hung, 2010) and anti-apoptosis (Krauss, 2003). Besides receptor tyrosine kinase pathways, Akt can be activated by platelet derived growth factor receptor (PDGF-R), insulin, epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), and insulin-like growth factor I (IGF-I) (Liao & Hung, 2010). This pathway is stimulated by several growth factors, cytokines, tumour necrosis factor, estrogen and other factors. Similar to ERK1/2 MAPK, this pathway also has a distinct anti-apoptotic function and promotes cell survival (Krauss, 2003). Altered Akt activity has been associated with endometriosis. However, comparing to MAPK pathways, the published data on the role of PI3K/Akt pathway in endometriosis is very limited. Recently, Zhang and colleagues have determined E2 induced endometriotic epithelial cell proliferation involved PI3K/Akt signaling pathway (Zhang *et al.*, 2010).

1.6 Roles of apoptosis in endometriosis

1.6.1 An overview of apoptosis

Apoptosis, or programmed cell death is an active process to eliminate unwanted, damaged or infected cells (Demedts *et al.*, 2006). Morphological changes of apoptotic cells include cell shrinkage, membrane blebbing, chromatin condensation, formation of apoptotic bodies and activation of caspases (Harada *et al.*, 2004; Conde de la Rosa *et al.*, 2006). In mammals, signaling cascades contributing to apoptotic cell death are divided into two main pathways, as shown in *Figure 1.20*: i. "*intrinsic*" (The mitochondrial pathway) that is activated from within the cell, ii. "*extrinsic*" (Death receptor-triggered apoptosis) that is initiated by external ligands that bind to and activate transmembrane receptors. The intrinsic pathway

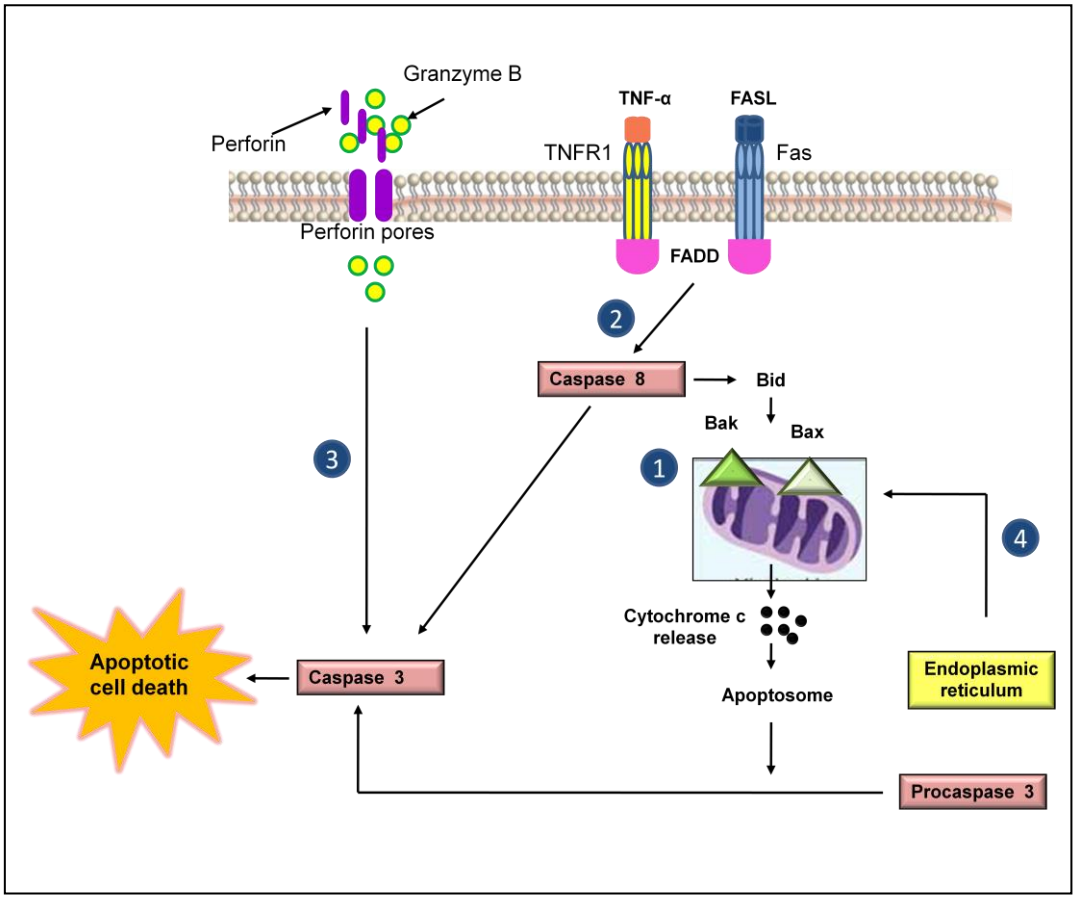


Figure 1.20: Different pathways involved in apoptosis.

***Intrinsic pathway*: Mitochondrial dependent pathway (1)**: Mitochondria release cytochrome c. Together with Apaf-1 and procaspase 9, cytochrome c will form the apoptosome complex that is able to cleave and activate procaspase 3

***Extrinsic pathway*: Ligand death-receptor pathway (2):** Fas ligand (FasL) and tumour necrosis factor (TNF) trigger apoptosis by binding on *'death receptors'*, Fas and Tumour Necrosis Factor Receptor 1 (TNFR1). Activated receptors recruit the adaptor protein FADD which further activate Caspase 8 and directly activates caspase 3 or cleaves Bid. **Cytolytic effector cell pathway:** Cytotoxic T cells release granzyme B and perforin. Perforin forms transmembrane pores that facilitate granzyme B to enter the cells, which then activate caspase 3

***Endoplasmatic reticulum pathway* (4):** The endoplasmic reticulum can also induce apoptosis via stimulation of the mitochondrial pathway.

involves death-promoting stimuli from the cellular compartments that favor mitochondrial membrane permeabilization (MMP) whereas the extrinsic pathway involves the lethal signal comes from the extracellular microenvironment and is transduced within cells (Galluzzi *et al.*, 2007). Disruption of the MMP is one of the earliest intracellular events that occur following the induction of apoptosis (Wang *et al*., 2010). There is another pathway that has been proposed, the endoplasmatic reticulum pathway (Demedts *et al.*, 2006), as shown in *Figure 1.20*.

1.6.1.1 Intrinsic pathway: The mitochondrial dependent pathway

Various stresses have been shown to trigger apoptosis through this pathway including elevated ROS, UV radiation, protein kinase inhibition and growth factor deprivation (Krauss, 2003). In the presence of apoptotic stimuli, the pro-apoptotic protein/s of the Bax/Bak group translocate from the cytosol to the outer membrane of the mitochondria, which allows the release of different mitochondrial proteins that are normally located in the intermembrane. Of the proteins, cytochrome c is the most important for triggering further downstream apoptotic events. The translocation of pro-apoptotic proteins leading to conformational changes within the outer mitochondrial membrane forming a pore-like structure that facilitates escape of cytochrome c from the mitochondria. Cytochrome c will then assembles with Apaf1 and procaspase 9 to form the apoptosome, which is able to cleave and activate procaspase 3 to form caspase 3 and leads to apoptotic cell death (Krauss, 2003; Demedts *et al.*, 2006). The changes in the mitochondria results in mitochondrial membrane permeabilization leading to apoptotic cell death.

1.6.1.1.1 Caspases

Caspases, a family of proteases are central in the initiation and execution of the apoptotic program (Krauss, 2003). The name caspase is a contraction of cysteine-dependent, aspartate-specific protease. To date, 14 mammalian caspase sequences

have been reported, namely caspases 1-14. Functionally, caspases are grouped into two biologically distinct subfamily; i. Initiator caspase, which mediates initiation of apoptosis including caspase 8 and 9, and ii. Executioner caspases, which carry out the apoptosis including caspase 3, 6 and 7.

1.6.1.1.2 The family of Bcl-2 proteins

Bcl-2 family members are the key regulators that control the mitochondrial pathway. To date, more than 20 members of the Bcl-2 family have been identified, which can have a negative or a positive effect on the initiation of the apoptotic program (Krauss, 2003). Bcl-2 family members possess up to four conserved Bcl-2 homology domains designated BH1, BH2, BH3, and BH4. Based on the structure and function, the Bcl-2 family has been divided into three groups (Gross *et al.*, 1999; Cory & Adams, 2002):

Group I: Consists of BH domains 1-4. All members of this group have anti-apoptotic functions such as Bcl-x_L

Group II: The structure is similar to group I, but lack the N-terminal BH4 domain. They have pro-apoptotic functions with examples; Bax and Bak proteins

Group III: Consists of large proteins that contain a single BH3 domain. They act by binding to group I and/or II family members via their BH3 domain and thought to be sensors of diverse pro- and anti-apoptotic stimuli, and integrating these into life-or-death decision. Some examples are Bid, Bad, Bim, Noxa and Puma.

1.6.1.2 Extrinsic pathway

1.6.1.2.1 Ligand death-receptor-pathway

This pathway is activated by extracellular signals and is mediated by binding of the members of the tumor necrosis factor (TNF) family (i.e Fas ligand and TNF-α) to

death receptors on the cell surface, namely Fas (also known as CD95) and tumor necrosis factor (TNF)-α receptor 1 (TNF-R1) (Krauss, 2003; Demedts *et al.*, 2006), as shown in *Figure 1.20*. The activated death receptors recruit the adaptor protein FADD (Fas-Associated Death Domain), which further recruit procaspase 8 (Muzio *et al.*, 1998). After cleavage, the mature caspase 8 then directly activates caspase 3 or cleaves Bid. Truncated Bid (tBid) interacts with Bax and Bak, which form a pore in the outer mitochondrial membrane through which cytochrome c is released (Demedts *et al.*, 2006; Galluzzi *et al.*, 2007).

1.6.1.2.2 Cytolytic effector cell pathway

This pathway is also known as granzyme B/perforin system. It is mainly exerted by circulating leukocytes including cytotoxic T Lymphocytes (CTL) and natural killer (NK) cells. Both granzyme B and perforin are stored in secretory granules inside leukocytes. This apoptotic pathway involves release of cytotoxic granule-stored effector molecules, granzyme B, upon specific interaction with a target cell. Perforin, a granule protein form transmembrane channels that facilitate delivery of granzymes B into the cells leading to apoptosis, via activation of caspase 3 (Saito *et al.*, 2011), as shown in *Figure 1.20*.

1.6.1.3 Endoplasmatic reticulum pathway

This pathway is activated as a reaction to stress, either directly stimulating the mitochondrial pathway *(Figure 1.20)* or through caspase 12. This caspase has been reported to be involved in response to hypoxia (Demedts *et al.*, 2006). However, there is very limited published data on this apoptotic pathway.

The different initiation pathways (intrinsic, extrinsic and endoplasmatic reticulum pathways) converge further downstream into activation of caspase 3. Susequently, the effector caspase 3 cleaves ICAD (inhibitor of CAD) and releases it from CAD (caspase-activated DNAase). CAD translocates from the cytoplasm to the nucleus and can now act as active endonuclease and fragment DNA.

1.6.2 Apoptosis resistance is responsible for endometriosis progression

Numerous authors have agreed that the imbalance between cell proliferation and apoptosis plays an important role in the pathogenesis of endometriosis (Dmowski *et al.*, 2001; Braun *et al.*, 2002). The percentage of apoptosis in endometrial cells was significantly reduced among women with endometriosis as compared to normal women, which implying that the surviving cells that enter the peritoneal cavity is higher in women who develop endometriosis (Gebel *et al.*, 1998). Moreover, it has also been reported that the apoptotic index in endometrial epithelial cells was significantly lower in women with endometriosis as compared to normal women (Dmowski *et al.*, 2001). Interestingly, these authors have found a trend toward a decrease in apoptosis index with increasing stage of the disease.

The data on apoptosis studies in endometriosis suggest that the decrease in apoptosis facilitates ectopic survival and proliferation of endometrial cells that retrogradely enter the peritoneal cavity. Some drugs that used to control endometriosis act by increasing apoptotic cells (Gomes *et al.*, 2009). For example, the used of combined oral contraceptive for one month significantly reduced cell proliferation and increased the apoptotic index in the eutopic endometrium of patients with endometriosis as compared to without endometriosis (Imai *et al.*, 2000). With the basic concept of inducing apoptosis inhibits cell growth, it has to be determined whether any compound which can induce apoptosis can be proposed as potential therapy in endometriosis.

1.7 Aims of the project

Despite decades of study, some of the features of endometriosis including its epidemiology, pathogenesis, ideal treatment modalities and its association with infertility, remain obscure. The above review summarizes recent progress in our understanding of the pathogenesis of endometriosis that involved various factors. Although the *'retrograde menstruation'* theory is widely accepted as the cause of disease development, factors that induce endometriotic cell growth and proliferation are still controversial. In recent years, the roles of estrogen and ROS have been implicated in endometriosis. However, it is still uncertain whether ROS cause endometriotic cell proliferation or whether endometriosis induces elevated ROS. Although endometriosis is known as an *'estrogen-dependent'* disease, the precise molecular basis of estrogen action on cellular proliferation remains obscure. The association between ROS and steroid hormones has not been established yet. Various estrogen biosynthesis enzymes have been described, however their roles in endometriosis are still controversial. Cell signaling pathways have been implicated in the mechanism of cell proliferation, but whether any of these pathway/s are involved in the mechanism of endometriotic cell proliferation is still debatable. The ultimate aim of unravelling these complex pathways is to develop novel treatment strategies for endometriosis. Since ROS have been implicated in the pathogenesis of endometriosis, it is to be determined whether antioxidants can be used as an alternative or auxiliary treatment in addition to the current drug therapy.

Hypotheses:

1. Oxidants and lipid peroxidation products (LPP) induce proliferation of endometriotic cells, whereas antioxidants may limit proliferation

2. Additive or synergistic effects between estradiol and oxidants or LPP may aggravate endometriotic cell proliferation

3. The up-regulation of estrogen biosynthesis enzymes are responsible for the progression of endometriotic cell growth and proliferation

4. Signaling pathway/s mediate the mechanism of endometriotic cell proliferation

5. The expression of antioxidant enzyme/s can be used as potential biomarkers in the pathogenesis of endometriosis

The general objectives of this study are:

1. To evaluate the effects of oxidants (hydrogen peroxide and menadione) and lipid peroxidation products (LPP) acrolein in mediating endometriotic cell proliferation in endometriotic cell line

2. To determine whether estradiol alone or in combination with oxidants or acrolein produce different effects on endometriotic cell growth

3. To demonstrate changes in gene expression of estrogen biosynthesis and metabolism enzymes in endometriotic cells caused by estradiol, oxidants, acrolein or combination of estradiol with oxidants or acrolein

4. To elucidate the mechanism of signaling pathways, (MAPK/ERK1/2 and PI3K/Akt) that may be present in the mechanism of endometriotic cell proliferation

5. To determine whether antioxidant (N-acetylcysteine) can inhibit endometriotic cell proliferation, which can be proposed as an alternative treatment in endometriosis

6. To determine antioxidant enzymes (heme oxygenase-1 and superoxide dismutase 1) expressions in endometriotic cells and their potential as a biomarker in the pathogenesis of endometriosis

7. To determine levels of aromatase and heme oxygenase-1 in women with endometriosis and without endometriosis by measuring the mRNA levels of endometriotic and endometrial tissue respectively.

CHAPTER TWO:

GENERAL MATERIALS

AND

METHODS

2.1 Materials

2.1.1 Endometriotic cell line

Immortalized human endometriotic epithelial cell, 12-z cell line at passage 15 were gifts from Professor Anna Starzinski-Powitz, Frankfurt University, Germany. The 12-z cell line was generated from primary peritoneal human epithelial endometriotic cells through immortalization by *in situ* electroporation with SV-40 T-antigen (Zeitvogel *et al.*, 2001). 12-z cells have been confirmed to have retained phenotypic characteristics (Banu *et al.*, 2008). The expression of several genes associated with steroid biosynthesis and signaling, cell cycle regulation, extracellular matrix degradation, angiogenesis, cell growth and survival, cytokine production, PGE_2 biosynthesis and much more have been studied and the gene expression pattern in 12-z cells closely match those *in vivo* from women with endometriosis and endometriosis animal models (Banu *et al.*, 2008).

2.1.2 Human samples

Total RNA isolated from human tissue samples were gift from Professor Tea Lanisnik Rizner from University of Ljubljana, Slovenia. The tissues were obtained during surgery from 6 patients with ovarian endometriosis and 5 samples of controls are from women with *Uterine myomatosus* or fibroid*s*. The details of the endometriosis patients are shown in *Table 2.1*. The sample collection was approved by the National Medical Ethics Committee of the Republic of Slovenia. Total RNA was extracted from each sample.

Table 2.1: Details of endometriosis patients

Age	Diagnosis	Phase of menstrual cycle
24	*Endometriosis ovarii*	Proliferative
27	*E. ovarii*	Early secretory
34	*E. ovarii*	Proliferative
32	*E. ovarii*	Secretory
40	*E. ovarii*	Proliferative
39	*E. ovarii*	Late proliferative

Samples were obtained from 6 patients with ovarian endometriosis during surgery.

2.1.3 Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM), Penicillin-Streptomycin (10,000 units penicillin; 10 mg streptomycin/ml 0.9% NaCl) Solution, Fetal Bovine Serum (FBS), Trypsin-EDTA Solution (1x), Sodium Pyruvate (11.0 mg/ml sodium pyruvate in tissue culture water), dimethyl sulphoxide (DMSO), Dulbecco's phophate buffered saline (DBPS), hydrogen peroxide (H_2O_2), menadione, acrolein, N-Acetylcysteine and 17β-estradiol were obtained from Sigma-Aldrich (UK). 3- (4, 5-Dimethylthiazol-2-yl) -2, 5-diphenyltetrazolium bromide (MTT) was obtained from Sigma-Aldrich (Germany). Bio-Rad Protein Assay Reagent was obtained from Bio-Rad Laboratories (Hertfordshire, UK). Acrylamide was obtained Kramel (Cramlington, UK). Tris base and NaCl were obtained from Fisher Scientific (UK), Ethylenediaminetetraacetic acid disodium salt (EDTA) was obtained from BDH Chemicals limited (Poole, England) and HCl was obtained from Reidd-de Haeu (Germany).

In vitro Toxicology Assay Kit (Lactate Dehydrogenase Based) was obtained from Sigma-Aldrich (UK), cat no:TOX-7. SOD determination assay kit (Product Number 19160) was obtained from Fluka (UK). Annexin VCy3 Apoptosis Detection Kit Plus (Cat. No K202-25) was obtained from BioVision, UK. Estrone EIA kits were purchased from ALPCO Diagnostics, USA and PGE_2 EIA kits were purchased from Cayman Chemical, UK.

2.1.4 Cell Culture plastic wares and equipments

Tissue culture flasks: T-75 cm^2 and T-175 cm^2 flasks, 6-well and 24-well plates were obtained from Fisher Scientific (UK) whereas 96-well plates were obtained from Greiner Bio-one (UK). Cell scrapers were obtained from BD biosciences (UK).

2.1.5 Antibodies

The antibodies used for Western blotting are listed in *Table 2.2*.

2.1.6 Oligonucleotides

The oligonucleotide primers used for Polymerase Chain Reaction (PCR) are listed in *Table 2.3*.

Table 2.2: List of antibodies used

Antibodies	Source	Dilution	Cat. No
Aromatase	A gift from Dr. Hilary Carswell, University of Strathclyde	1:3000	-
COX-2	Cell Signaling, UK	1:1000	4842S
ER-α	Santa Cruz Biotech. Inc	1:1000	sc130072
ER-β	Santa Cruz Biotech. Inc	1:1000	sc56828
GAPDH	Santa Cruz Biotech. Inc	1:3000	sc25778
Tom40	Santa Cruz Biotech. Inc	1:3000	sc11025
Lamin b	Santa Cruz Biotech. Inc	1:3000	sc6216
SOD1	Santa Cruz Biotech. Inc	1:3000	sc11407
HO-1	Santa Cruz Biotech. Inc	1:3000	sc7695
Bax	Santa Cruz Biotech. Inc	1:3000	sc493
Caspase-3	Santa Cruz Biotech. Inc	1:3000	sc7148
Cytochrome-C	Santa Cruz Biotech. Inc	1:3000	sc7159
pJNK	Cell Signaling, UK	1:3000	4671S
JNK	Cell Signaling, UK	1:3000	9252S

Antibodies	Source	Dilution	Cat. No
Phospho-p44/42 MAPK (ERK1/2)	Cell Signaling, UK	1:3000	9102S
p44/42 MAPK (ERK1/2)	Cell Signaling, UK	1:3000	4695S
Phospho-Akt (Ser473)	Cell Signaling, UK	1:3000	4058S
Akt	Cell Signaling, UK	1:3000	9272S

2.2 Cell Culture Methods

2.2.1 12-z cell line

Cells were maintained in Dulbecco's minimum Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% antibiotics (penicillin and streptomycin) and 1% sodium pyruvate using T-75 cm^2 flasks. Cells were cultured in an incubator with a humidified atmosphere of 5% CO_2/ 95% air at 37 °C. When cells had reached 70-80% confluence, they were further passaged or plated out into wells according to the requirements of the experiment using Trypsin-EDTA Solution (1x), or frozen down for storage at -80 °C for later use. For splitting, before Trypsin-EDTA treatment for about 2 min, existing medium was discarded and the adherent cells were washed with Dulbecco's phophate buffered saline. The pellet that was obtained after spinning was resuspended with new media and transferred

Table 2.3: Oligonucleotide Primers

Primer	Sequence
AKR1C3 forward	5'-GTAAAGCTTTGG-AGGTCAC-3'
AKR1C3 reverse	5'-CACCCATCGTTTGTCTCGT-3'
ER-α forward	
ER-α reverse	Catalog no: QT 00044492
ER-β forward	
ER-β reverse	Catalog no: QT 00060641
17β-HSD type 1 forward	5'-GTACGTCTGGCTTCAGATCCATCC-3'
17β-HSD type 1 reverse	5'-TATTGGTAGAAGCGGTGGAAGG-3'
17β-HSD type 2 forward	5'-GGGGAGCTTCTTCTTATGACTG-3'
17β-HSD type 2 reverse	5'-TCCGCTGTGCTAAGATGTAGTCC-3'
HO-1 forward	5'-TGA TAG AAG AGG CCA AGA-3'
HO-1 reverse	5'-TTT CCA GAG AGA GGG ACA-3'
SOD-1 forward	5'-AGG GCA TCA ATT TCG AGC-3'
SOD-1 reverse	5'-GCC CAC CGT GTT TTC TGG A-3'
GAPDH forward	5'-GGA GTC AAC GGA TTT GGT-3'
GAPDH reverse	5'-GTG ATG GGA TTT CCA TTG-3'

GAPDH, AKR1C3, 17β-HSD type 1 and 17β-HSD type 2 oligonucleotides were purchased from Eurofins MWG operon (UK) and other oligonucleotides were purchased from Qiagen, UK.

into new flasks. Cells were splitted with the split ratio was 1:3. In order to freeze cells, they were suspended in 90% FBS and 10% dimethyl sulphoxide (DMSO). All cells used in this study are at low passage numbers (<25). The cell culture procedures were done in the culture hood that was supplied by ICN Gelaine (England).

2.2.2 Cell Count

10 µl of resuspended cell suspension was placed into a Bright-line hemocytometer (Sigma-Aldrich-Germany) and counted using the microscope (Olympus CK40 from Olympus (Japan). Cells were countered in 4 outer quadrants and the mean was taken. The cell number was calculated as below:

Cell number per ml = Average cell count in 4 squares x 10^4

The cell suspension was then diluted and obtained cell number was taken according to the requirements of the experiment.

2.2.3 Treatment of cells

2.2.3.1 Treatment of cells for MTT assay

To determine the effects of compounds on cell growth and proliferation, cells were plated in 96-well plate at a density of 5 x 10^3 cells per well for 24 h. Cells were treated with oxidants (0.5 µM-500 µM hydrogen peroxide and 10 µM-35 µM menadione), lipid peroxidation product acrolein: 10 µM-100 µM or 10^{-11} M-10^{-6} M 17β-estradiol at 24 h or 48 h. To determine whether estradiol with oxidants or acrolein gives further proliferation, cells were pre-treated with estradiol for 24 h followed by addition of oxidants or acrolein for 48 h. These compounds are used in most of the experiments in this study.

To determine the inhibitory effects of compound of cell growth, cells were plated in 96-well plate at a density of 5 x 10^3 cells per well for 24 h. Subsequently, cells were treated with antioxidant 0.5 mM-30 mM N-acetylcysteine for 24 h.

2.2.3.2 Treatment of cells for total glutathione assay

Cells were cultured in 6-well plates for 24 h. Cells were pre-treated with or without N-acetylcycteine for 24 h followed by addition of hydrogen peroxide for 48 h. Cells were washed and scraped into PBS and and centrifuged at 1000 rpm for 5 min at 4 ^{o}C. The pellet was re-suspended in PBS and protein concentrations of the samples were measured by Bradford's assay and normalised in 5% SSA.

2.2.3.3 Treatment of cells for Fluorescence imaging for apoptosis and necrosis

Cells were plated in 6 well plates at a concentration of 1x 10^5 cells/well and allow to attach for 24 h. Cells were then treated with antioxidant, NAC at different concentrations for 24 h.

2.2.4 Cell viability assay

2.2.4.1 MTT assay

Principle

Cell viability was assessed by the [(3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] (MTT) assay method (Mosmann, 1983). This assay is a standard colometric assay, which measures changes in colour where there is conversion of soluble yellow coloured tetrazolium salt into an insoluble purple coloured formazan. The conversion is carried out by succinate dehydrogenase in

the mitochondrion and cytosol of viable cells. A solubilizer, DMSO is added to dissolve the insoluble purple formazan product into a colored solution. The number of surviving cells is directly proportional to the level of the formazan product created. The coloured compound formed has a characteristic absorption at 560nm wavelength.

Procedures

For the MTT assay, cells were plated in 96-well plates in triplicate at cell densities of 5000 – 10 000 cells per well and allowed to attach for 24 h. Subsequently, the test compound was added to each respective well and incubated according to the experimental design. At the end of the incubation period, 20 µl of MTT solution in growth medium was added to each well (final concentration: 1.2 mg/ml) and incubated for 4 h. Then, growth medium was carefully removed by pipetting. The formazan crystals were dissolved in 100 µl DMSO after agitation in shaker for 10 min. Then, the absorbance was measured using a microtiter plate reader at optical density of 560nm using the Labsystems iEMS Reader MF (supplied by Labsystems and Life Sciences International Limited, UK) and the background values were subtracted. Results were expressed as mean value of percentage of untreated control cells.

Percentage of control = (OD of the samples/OD of the control) x 100

2.2.4.2 Lactate Dehydrogenase assay

Principle

The Lactate Dehydrogenase assay (LDH) assay measures the release of the intracellular enzyme LDH upon damage of the plasma membrane, and an increase in LDH release can be an index of cell damage (Fotakis & Timbrell, 2006). The method was based on the reduction of NAD by the action of LDH. The resulting

reduced NAD (NADH) is used to convert a tetrazolium dye into a coloured compound which can be monitored spectrophotometrically. Changes in absorbance were monitored at 490nm and background absorbance was monitored at 690nm.

Procedures

Cells were plated in 24-well plates and allowed to attach for 24 h. Subsequently, test compound was added to each respective well and incubated according to the experimental design. At the end of the incubation period, one aliquot of medium (0.2 ml) was taken out to measure the extracellular LDH leakage. Then, LDH Assay lysis solution was added and incubated for 45 min. Subsequently, the plate was centrifuged at 250 x *g* for 4 min to pellet cells. The supernatant was transferred to clean flat-bottom plate, which provides a measure of total cell activity. Assay mixture was prepared that consists of LDH Assay Substrate Solution, LDH Assay Cofactor and LDH Assay Dye Solution with the ratio 1:1:1. 30 µl of the sample supernatant and 60 µl of the LDH assay mixture were added to each respective well. The plate was covered with aluminium foil and incubated at room temperature for 20-30 min. The reaction was terminated with 10 µl 1N HCl. Absorbance was measured using Labsystems iEMS Reader MF (Microplate reader) at 490nm with background absorbance at 690nm. The background absorbance was subtracted from the absorbance at 490nm. Results were presented as mean value of percentage of control. The ratio between the LDH activity in culture medium and the total LDH activity was calculated to determine the percentage of LDH leakage for each sample.

2.3 Preparation of cell extracts

2.3.1 Preparation of protein extracts for enzyme assays

The following solutions were prepared:

- TEN Buffer (Tris.Hcl-EDTA-NaCl)
  Tris-HCl (80 mM) at pH 7.5, EDTA (10 mM) at pH 8.0 and 1 M NaCl
- Tris-HCl (250 mM) pH 7.5

Freeze-Thaw method

Cells were prepared by 'freeze-thaw' lysis method to preserve enzyme activity. Cells were plated in 6-well plates. To prepare the samples, the media was aspirated and cells were washed in PBS followed by resuspension in 300 µl lysis buffer (section 2.3.3). Cells were allowed to sit on ice for 5 min and scraped. The scraped cells were transferred into eppendorf tube and centrifuged at 10 000 x *g* for 1 minute. Then, the supernatant was removed and the pellet was resuspended in 100 µl 250 mM Tris-HCl (pH 7.5). The cell suspension was frozen at -80 ^{o}C for 5 min and thawed rapidly at 37 ^{o}C for 5 min. The freeze-thaw procedure was repeated 5 times. The cell lysate was then centrifuged at 1000 x *g* for 5 min and the supernatant was stored at -20 ^{o}C. Protein concentrations were determined by using Bradford's method against BSA standard curve.

2.3.2 Preparation of protein extracts for Western blotting

Cells were cultured in 6-well plates at cell densities of 1-2 x 10^5 cells per well and allowed to attach for 24 h. Subsequently, test compound was added to each respective well and incubated according to the experimental design. After the incubation period, cells were washed with Phosphate Buffer Saline (PBS - 137 mM

NaCl, 10mM sodium phosphate, 2.7mM KCl, pH 7.4) and scraped in 100 µl of 2 x LSB (Laemmli Sample Buffer). Samples in 2 x LSB were boiled for 5 min at 100 °C to denature the proteins and centrifuged at 13 000 x *g* for 30 seconds. To prepare 2 x LSB, a mixture of 3.13 ml 1M Tris-HCl at pH 6.8, 2 g SDS, 9 ml Glycerol and 5 ml 2-Mercaptoethanol was prepared in 32 ml dH_2O. 2% Bromophenol blue was added freshly to each sample on the day of running the samples.

2.3.3 Subcellular fractionation

The following solutions were prepared:

- Homogenising Buffer:

500 ml of homogenising buffer was prepared with sterile dH_2O with a mixture of final concentrations of each chemical; 20 mM HEPES-KOH at pH7.5, 10 mM Sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 2 mg/ml Aprotinin, 10 mg/ml Leupeptin and 5 mg/ml Pepstatin

- Lysis Buffer:

50 mM Tris-HCl at pH7.4, 150 mM NaCl, 0.5% Triton X-100, 20 mM EGTA, 1 mM DTT and 1 mM Sodium orthovanadate.

Cells were plated in 3 x 175cm^2 tissue culture flasks and were allowed to grow to 80% confluent. Cells were then treated with test compound and incubated according to the experimental design. After the incubation period, cells were washed with ice-cold PBS and scraped. The scraped cells were then centrifuged at 1,000 x g for 3 min at 4 °C and the pellets were resuspended in 1 ml ice cold homogenizing buffer and homogenised with the homogenizer-Model T25basic (from IKA Labortechnik, UK) in order to break the cell membrane to release the cell contents. The cell homogenate was centrifuged in Beckman centrifuge-

AvantiTMJ-30I (from Beckman) at 3,000 x *g* for 5 min at 4 °C and the pellets were retained and resuspended in the lysis buffer as the nuclear fraction and stored at -70 °C until used for Western blotting. The supernatant was removed and centrifuged at 10,000 x *g* for 10 min at 4 °C and the pellets obtained were resuspended in lysis buffer and retained as the mitochondrial fraction. The fraction was stored at -70 °C until further use. The supernatant was removed and centrifuged further at 100,000 x *g* for 1 h at 4 °C. The supernatant was removed and retained as the cytosolic fraction. 100 µl of the cell fraction was mixed with 200 µl of the 2 x LSB and boiled in a waterbath for 5 min at 100 °C to denature the proteins and centrifuged at 13 000 x *g* for 30 seconds.

2.4 Determining protein concentration – Bio-Rad Protein Assay

The Bio-Rad Protein assay is based on the method of Bradford (Bradford, 1976). The method was based on the formation of dye-protein complex solution between Bradford reagent and protein with a characteristic absorbance at 595nm. Several serial dilutions of Bovine Serum Albumin (BSA) protein standard, ranging from 0-20 µg/ml were made in distilled water and made up to 800 µl in eppendorf tubes. Subsequently, 200 µl of Bio-Rad reagent was added and the absorbance measured at 595nm. A standard curve of OD_{595} *versus* concentration of protein standard was plotted. To determine the protein concentration of each sample, the reaction mix was prepared that consist of 10 µl of sample and 790 µl of distilled water and 200 µl of Bio-Rad reagent. Unknown protein concentrations were calculated from the standard curve.

2.5 Gel electrophoresis of proteins

Principle

Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) coupled with Western blotting is mainly used to identify the presence of a given protein. In SDS-PAGE, proteins are separated based on their molecular weight in an applied electric field or known as electrophoresis (Weber & Osborn, 1969). To migrate through the gel, the proteins are first denatured and negatively charged when they were exposed to a detergent such as SDS. Subsequently, the denatured proteins travel along the polyacrylamide gel according to their molecular weight. The different lanes of bands were separated based on the individual components sizes that can be identified by a molecular weight marker.

2.5.1 Equipment and buffers

To separate proteins based on their molecular weight, one dimensional electrophoresis on SDS-acrylamide gels was used. Electrophoresis was performed using the Atto mini gel system from Genetic Research Instrumentation Ltd., Dunmow, Essex, UK. Samples were run at 125V and 200mA for 90 min. The buffer system used was described previously by Laemmli (Laemmli, 1970).

Buffers preparation:

<u>4 x Resolving Buffer (Bottom Gel)</u>:

Tris base, 90.8 g; SDS, 2 g. 450 ml of dH_2O was added and the pH was adjusted to 8.8 with concentrated HCl. The solution was made up to 500 ml with dH_2O and then filter sterilised.

4 x Stacking Buffer (Top Gel):

Tris base, 15.14 g; SDS, 1 g. 200 ml dH_2O was added and the pH was adjusted to 6.8 with concentrated HCl. The solution was made up to 250 ml with dH_2O and then filter sterilised.

10 x Running buffer

Tris base, 30.28 g; Glycine, 144 g; SDS, 10 g; made up to 1 litre with dH_2O

1x Running buffer

100 ml of 10 x Running buffer was made up to 1 litre with dH_2O

Gel preparation:

The composition of gels was as follows:

Resolving Gel (10% Gel):

30% Acrylamide and bis-acrylamide solution, 6.6 ml; 4 x Resolving Buffer, 5.0 ml; Ammonium persulphate (100 mg/ml freshly prepared), 100 µl; TEMED, 10 µl; dH_2O 8.2 ml.

Stacking Gel (5%Gel):

30% Acrylamide and bis-acrylamide solution, 1.64 ml; 4 x Stacking Buffer, 2.5 ml; dH_2O, 5.86 ml; Ammonium persulphate (100 mg/ml), 60 µl; TEMED, 10 µl.

2.5.2 Sample Loading and Electrophoresis

Samples were boiled in 2 × LSB at 100 °C for 5 min to denature the proteins and approximately 10 µg protein was loaded in the well.

2.6 Western Blotting

Principle

The Western blot or immunoblot is a detection method of specific proteins in biological samples (Heidebrecht *et al.*, 2009). The proteins that have been separated by gel electrophoresis are transferred to a membrane (nitrocellulose membrane or polyvinylidene difluoride-PVDF) and probed using specific primary antibody to the target protein and secondary enzyme labeled antibody.

Buffers and Solutions

1 x Transfer Buffer:

Glycine, 14.4 g; Tris base, 3 g; 800 ml dH_2O and made up to 1 litre with methanol.

10 x TBS (Tris-buffered Saline):

1 M Tris-HCl pH 7.5, 100 ml; 4M NaCl, 375 ml.

1 x TBS/Tween (0.2% Tween):

10 x TBS, 100 ml; Tween 20, 2 ml; made up to 1 litre with dH_2O.

Blocking Solution (10%):

5 g dried skimmed milk powder (Marvel); made up to 50 ml with 1 x TBS/Tween.

2.6.1 Transfer and Detection

The gel, nitro-cellulose paper, Whatman paper and sponges were soaked in 1 x Transfer Buffer. A western transfer apparatus is prepared by sandwiching a piece of sponge, two pieces of Whatman paper, the nitro-cellulose membrane, the SDS-

PAGE gel, two more pieces of Whatman paper and another piece of sponge. The air bubble in between the layers was removed. The assembled sandwich was then placed in transfer tank (Bio-Rad gel apparatus) filled with 1 x Transfer Buffer. Transfer to nitrocellulose was carried out for 1 h and 30 min at 250 mA. Subsequently, the nitrocellulose membrane was removed into a clean plastic container containing the blocking solution, in TBS/Tween (20 mM Tris-HCl/150 mM NaCl/0.01% (v/v) Tween) containing 10% (w/v) skimmed milk and left for 1 h to block the protein-binding sites present on the membranes. The nitrocellulose membranes were probed for overnight with primary antibodies in TBS/Tween-skimmed milk. Membranes were washed with TBS/Tween (4 x 10 min) and probed with horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit IgG at 1:3000 dilution in TBS/Tween-skimmed milk) for 2 h. Membranes were washed again with TBS/Tween (4 x 10 min) and antibodies detected using enhanced chemiluminescence (ECL; Amersham).

Antibodies:

Horseradish peroxidase-conjugated secondary antibody, goat anti-rabbit IgG was supplied by Bio-Rad Laboratories (Hertfordshire, UK).

2.6.2 Antibody Detection using Enhanced Chemiluminescence

Enhanced Chemiluminescence (ECL) substrate is an enhanced chemiluminescent substrate for detection of horseradish peroxidase (HRP) activity from antibodies and other Western blot probes.

Solutions:

<u>250 mM Luminol</u>

0.22 g Luminol (Fluka, cat no 09253) was dissolved in 5 ml DMSO. The solution was

kept in the dark at -20 °C in 500 µl aliquots.

90mM Coumaric acid

0.07 g Coumaric acid (Sigma, cat no C-9008) was dissolved in 5 ml DMSO and the solution was kept in the dark at -20 °C in 500 µl aliquots.

ECL 1 Solution

1 ml of 250 mM Luminol solution; 0.44 ml of 90 mM Coumaric acid solution; 10 ml of 1 M Tris-HCl (pH 8.5); was made up to 100 ml with dH_2O.

ECL 2 Solution

64 µl of H_2O_2 (30%); 10 ml of 1M Tris-HCl (pH 8.5); was made up to 100 ml with dH_2O.

Both solutions were kept in the dark at 4 °C. ECL reagents were prepared by mixing equal volumes of the two ECL solutions. The ECL working solution was poured on the membrane and exposed to get the images. Images were taken using a LAS-3000 luminescent image analyzer (Fujifilm). The band intensities were analysed using image J analysis software (www.imagejdev.org).

2.7 Measurement of Total Cellular Glutathione

Principle

Total intracellular glutathione (GSH) was performed according to methods by Eady and co-workers (Eady *et al.*, 1995). GSH interacts with DTNB (5, 5'-dithiobis-2-nitrobenzoic acid, Ellmant's reagent) by its sulfhydryl group and produces a yellow colored 5-thio-2-nitrobenzoic acid (TNB), which is measured at 412nm. Glutathione reductase reduces oxidised glutathione (GSSG) in the presence of NADPH. GSH is quantified by reference to a standard curve and expressed as µmol/mg protein. The coloured compound is proportional to the concentration of GSH. Preincubation of

the sample with the thiol masking agent 2-Vinylpyridine (2-VP) prevents measurement of GSH, resulting in measurement of GSSG only, which is also measured at 412nm.

Buffers and solutions

- Phosphate-EDTA dilution buffer (100 mM Na3PO4 – 1 mM EDTA, pH 7.4)
- 400 mM Sodium carbonate
- 5% Sulfosalicylic acid
- Reaction buffer: 1.9 U/ml Glutathione reductase and 0.4 mM β-Nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt (NADPH)
- 5-5'-Dithiobis (2-nitrobenzoic acid)(DTNB) [Ellman reagent]
- 200 mM Sodium carbonate-2.5% SSA buffer
- 2 M 4-Vinylpyridine

All chemicals were obtained from Sigma-Aldrich (Germany).

Assay procedure

To prepare GSH assay standard; 10 mg of GSSG was added to 10 ml of 5% SSA. Then, 10 µl of this mixture was added into 990 µl 5% SSA to develop 10 ng/ µl of GSSG stock. An amount of 100, 50, 40, 30, 20 and 10 µl of GSSG stock was added to labelled eppendorf tube and 200 mM Sodium carbonate-2.5% SSA buffer was added to make the total volume of 100 µl. This procedure will generate 10, 5, 4, 3, 2 and 1 ng/ µl GSH standards respectively. The assay was done using 96 well plates. 170 µl of Reaction buffer was added to each well whereby three wells were used for each measurement. Then, 20 µl of standard/ sample/ blank (200 mM Sodium carbonate-2.5% SSA buffer solution) was added to each respective well and incubated for 10

min in room temperature. Subsequently, 10 µl of DTNB substrate was added to each well and incubated again for 10 min in room temperature. After 10 min, the absorbance was read at 412nm. To determine oxidised GSSG, 1 µl of 2 M 4-VP was added to each sample and incubated for 1 h. The total GSH concentrations in the samples were determined against the GSH standard calibration curve. Reduced GSH was then calculated by substracting GSSG from the total GSH.

2.8 Enzyme assay

2.8.1 Superoxide Dismutase assay

Principle

The method was based on the ability of SOD to remove superoxide anion formed by xanthine oxidase (XO) that prevent it from converting water soluble tetrazolium salt (WST-1) (2-(4-Iodopheyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt), into a coloured compound, formazan (Peskin & Winterbourn, 2000). Absorbance of WST-1 was monitored at 450nm and is a measure of SOD activity. The rate of the reduction with O_2 is linearly related to the XO activity and is inhibited by SOD.

Assay procedure

The assay was carried using 96 well plates. The sample reaction well contains 20 µl cell extracts; 200 µl of WST and 20 µl of enzyme working solution. There are 3 types of blank wells, blank 1, 2 and 3, and only blank 2 contained sample solution. The sample blank reaction well contains 20 µl cell extracts; 200 µl WST and 20 µl of dilution buffer. The enzyme reaction well contains 20 µl double distilled water (ddH_2O), 200 µl of WST working solution and 20 µl enzyme working solution. The enzyme blank reaction well contains 20 µl ddH_2O, 200 µl WST and 20 µl of dilution

buffer. The plate was incubated for 20 min at 37 °C and absorbance was read at 450nm using a microplate reader. SOD activity was calculated as in the protocols and expressed as the rate of inhibition of WST reduction/mg protein.

SOD activity calculation

SOD activity in the cell extracts was calculated using the following equation:

SOD activity (inhibition rate %)

$$= \{[(Ablank_1 - A\ blank_3) - (Asample - Ablank_2)]/ (Ablank_1 - Ablank_3) \times 100$$

$Ablank_1$= Enzyme reaction mixture

$Ablank_2$ = Sample blank reaction mixture

$Ablank_3$ = Enzyme blank reaction mixture

Asample = Sample reaction mixture

2.9 Fluorescence imaging for apoptosis and necrosis

Principle

Soon after initiating apoptosis, cells translocate the membrane phospholipids, phosphatidylserine (PS), from the internal surface of the plasma membrane to the cell surface, which can be easily detected by staining with fluorescent conjugate of annexin V. The annexin V protein has a strong natural affinity for PS and so can be used in monitoring the translocation of PS to the external cell surface which is the characteristic of apoptotic cells. The SYTOX green dye is impermeable to live and apoptotic cells but gives an intense green fluorescence by binding to cellular nucleic acids. The cell populations can easily be distinguished by Fluorescence microscope using TRITC filter (red coloured filter) for Annexin VCy3 and FITC filter (green coloured filter) for SYTOX. Apoptotic cells show the red fluorescence indicate apoptotic cells whereas green fluorescence indicate necrotic cells.

Slides preparation

After cells were treated in 6-well plates, media were collected in 15 ml centrifuge tubes and cells were then detached by adding very low concentration of trypsin (0.1%) for 1 min. The detached cells were collected into the 15 ml centrifuge tube containing the aspirated media and then centrifuged at 1000 x *g* for 5 min. After centrifugation, the media were aspirated and the pellets were washed in PBS. Cells were counted and 1x 10^5cells were then collected by centrifugation at 1000 x *g* for 5 min. The collected cells were resuspended in 500 µl Binding Buffer. 5 µl of Annexin V-Cy3 dye and 1 µl of SYTOX Green dye were then added and incubated in the dark for 10 min at room temperature. After incubation, 50 µl of the mixture were mounted on a 0.8-1.0 mm Microscopic Slide and covered with coverslip. The slides were then placed upside down to allow for the uniform distribution of the cells and proceed to be viewed under the Fluorescence microscope.

2.10 RNA methods

2.10.1 RNA isolation

Total RNA was isolated from 12-z cells in 6-well plates using the SV Total RNA Isolation system from Promega, USA according to the manufacturer's instructions.

2.10.2 Determination of RNA Yield and Quality

RNA yield was determined spectrophotometrically at 260nm, where 1 absorbance unit (A260) equals 40 µg of single-stranded RNA/ml. RNA purity was also estimated spectrophotometrically from the relative absorbance at 230, 260 and 280nm (i.e., A260/A280 and A260/A230). A280 is the absorbance due to protein contamination and A230 is the absorbance due to DNA contamination. Pure RNA has an A260/A280 ratio between 1.7-2.1 and an A260/A230 ratio of 1.8-2.2.

2.10.3 Quantitative Reverse Transcriptase-Polymerase Chain Reaction (Q-RT-PCR)

2.10.3.1 Synthesis of First Strand Complementary DNA (cDNA) using Reverse Transcriptase

To develop cDNA, 5 µg of total RNA were mixed with 1 µg of random primer (500 µg/ml) and heated in 70 °C water bath for 5 min followed by incubation on ice for 2 min. Subsequently, the following components (obtained from Promega, UK) were added: 5 µl M-MLV Reverse Transcriptase Reaction Buffer (5x), 1.25 µl PCR Nucleotide Mix (10 mM), 6.75 µl Nuclease free water and 1 µl M-MLV Reverse Transcriptase (200 units/µl). The mixture was gently mixed by pipetting, incubated at 25 °C for 10 min and continued with thermal cycling. cDNA synthesis reactions were incubated at 42 °C for 50 min. Following incubation, the reaction was inactivated by heating at 70 °C for 15 min. First strand cDNA obtained from RNA samples was stored at -20 °C until use.

2.10.3.2 Quantitative Real Time-PCR

Q-RT-PCR was carried out using a set of oligonucleotide primers with the LightCycler instrument (Roche Diagnostics) with the specific cycling conditions for the LightCycler (*Table 2.4*). Reactions were set up in 20 µl volumes that consist of 5 µl of first strand cDNA, 1 µl of each primer (5 pmol/µl, final concentration of 0.25 µM), 3 µl of nuclease free water and 10 µl of PerfeCta SYBR Green Fastmix (obtained fromQuanta Biosciences). PerfeCTa SYBR green FastMix (2X) contains 2X reaction buffer containing optimized concentrations of $MgCl_2$, dNTPs (dATP, dCTP, dGTP, dTTP), AccuFast Taq DNA polymerase, SYBR Green I dye and stabilizers. This reagent

Table 2.4: The Q-RT-PCR parameters programmed for the LightCycler Instrument

Analysis Mode	**Cycles**	**Segment**	**Target Temperature**	**Hold Time**	**Acquisition Mode**
			Denaturation		
None	1	1	95 °C	30 s	None
			Amplification		
Quantification	45	Denaturation Annealing Extension	95 °C Primer dependent 72 °C	0 s 5 s Amplicon size/25	none none single
			Melting curve		
Melting curve	1	Denaturation Annealing Melting	95 °C 65 °C 0.1 °C /sec	0 s 15 s 0 s	none none continous
			Cooling		
None	1		40 °C	30 °C	none

is concentrated that contains all components, except primers and template for Q-PCR. Nuclease-free water was used as a negative control. The relative amount of cDNA synthesised in each RT-PCR was compared with GAPDH mRNA levels. Relative expression levels were calculated for each sample after normalisation to the GAPDH, using the ΔΔCT method for comparing relative fold-expression differences. The reproducibility of the quantitative measurements was evaluated by 3 independent cDNA syntheses.

2.11 Enzyme Immunoassay

2.11.1 Estrone EIA

Principle

This is a direct quantitative determination of estrone by enzyme immunoassay (EIA). The principle of the assay operates on the basis of competition between an unlabelled antigen (present in standards, control and samples) and an enzyme-labelled antigen (conjugate) for a limited number of antibody binding sites on the microwell plates. The washing and decanting procedures remove unbound materials. After the washing step, the enzyme substrate is added. The enzymatic reaction is terminated by addition of the stop solution and the absorbance is measured on the microplate reader. The intensity of the colour formed is inversely to the concentration of estrone in the sample. A set of standards is used to plot a standard curve. Subsequently, the amount of estrone in samples and controls can be read directly.

Buffers and solutions

- Rabbit Anti-Estrone Antibody Coated Microwell Plate-Break Apart Wells
- Estrone-Biotin Conjugate Concentrate
- Avidin-Horse Radish Peroxidase (HRP) Conjugate Concentrate

- Estrone Calibrators: 6 calibrators at different concentrations 0 pg/ml – 2000 pg/ml
- Control
- Wash Buffer Concentrate: the concentrated wash buffer was diluted 10 fold with deionized water before use.
- Assay Buffer
- TMB Substrate :containing tetramethylbenzidine and hydrogen peroxide in DMSO
- Stopping Solution : containing 1M sulfuric acid

To prepare working solution of the estrone conjugate; the estrone biotin and avidin HRP concentrate were diluted 100 fold each with assay buffer. For example: to a tube containing 2 ml of assay buffer, 20 µl of estrone-biotin and 20 µl HRP were added and mixed thoroughly.

Assay procedure

Briefly, a required number of microwells for a complete experiment were taken. An amount of 50 µl of calibrator, control and sample was added into corresponding labelled wells in duplicate. Then, 100 µl of the conjugate working solution was added into each well. The plate was covered with an adhesive strip and incubated on a plate shaker, approximately 200 rpm for 1 h at room temparature.

Thereafter, wells were aspirated and washed 3 times with 300 µl of diluted wash buffer per well and the plate was tap firmly against absorbance paper to ensure that it is dry. The tipping off/ wash step was repeated. Then, 150 µl of TMB was added into each well. Again, the plate was incubated on the similar plate shaker for 10-15 min at room temperature. Finally, the reaction was stopped by adding 50 µl of stopping solution into each well. The optical density readings were recorded

within 20 min after addition of the stopping solution, at 450nm using a microplate reader.

Calibrator curve:

A calibrator curve is shown in *Figure 2.1*. The estrone concentration of each sample was calculated using the equation that obtained from the calibrator curve. The concentration of the 'kit control' is recommended to be between 75-125 pg/ml and a value of 118 pg/ml was obtained in this experiment.

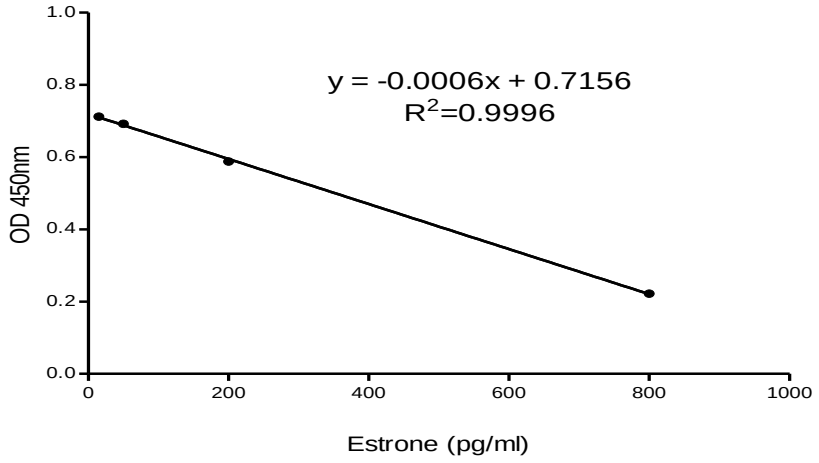


Figure 2.1: A calibrator curve to determine estrone concentrations.

2.11.2 Prostaglandin E_2 EIA

Principle

This assay is based on the basis of competition between Prostaglandin E_2 (PGE_2) and PGE_2-acetylcholinesterase (AChE) conjugate (PGE_2 Tracer) for a limited amount of PGE_2 Monoclonal Antibody. The amount of PGE_2 Tracer that is able to bind to the PGE_2 Monoclonal Antibody is inversely proportional to the concentration of PGE_2 in

the well, as the concentration of PGE_2 Tracer is held constant while the concentration of PGE_2 varies. This antibody-PGE_2 complex binds to goat polyclonal anti-mouse IgG that attached to the well. The plate is washed to remove any unbound reagents and Ellman's Reagent that contains the substrate to AChE is added to the well. A distinct yellow colour that absorbs strongly at 412nm is produced. Tracer bound to the well is inversely proportional to the amount of free PGE_2 present in the well during incubation.

Buffers and solutions

PGE_2 EIA kits were purchased from Cayman Chemical, UK. The kits contained:

- Precoated (Goat Anti-Mouse IgG) EIA 96-Well Strip Plate
- Tween 20
- EIA Tracer Dye
- EIA Antiserum Dye
- Ellman's Reagent
- EIA Buffer Concentrate (10X) : Dilute with 90 ml of UltraPure water
- Wash Buffer Concentrate (400X) : Dilute the 5 ml vial to 2 L and 1 ml Tween 20 was added
- Prostaglandin E_2 Express AChE Tracer
- Prostaglandin E_2 Express Monoclonal Antibody
- Prostaglandin E_2 Express EIA Standard: Standard was prepared at different concentrations (15.6, 31.3, 62.5, 125, 250, 500, 1000 and 2000 pg/ml)

Assay procedure

Several controls were used as follows:

Blank: background absorbance caused by Ellman's Reagent, which should be substracted from the readings of all other wells

Total activity (TA): total enzyme activity of the AchE-linked tracer

Non-specific Binding (NSB): non-immunological binding of the AchE-linked tracer to the well

Maximum Binding (Bo): maximum amount of tracer that the antibody can bind in the absence of free analyte

A required number of microwells for a complete experiment were taken. Various controls of the kit and samples were added to each respective well as in the *Table 2.5.*

Table 2.5: Pipetting summary of Prostaglandin E_2 EIA

Well	EIA Buffer	Standard/Sample	Tracer	Antibody
Blank	-	-	-	-
Total activity (TA)	-	-	5 µl (added after the first incubation)	-
Non-specific Binding (NSB)	100 µl	-	50 µl	-
Maximum Binding (Bo)	50 µl	-	50 µl	50 µl
Standard/Sample	-	50 µl	50 µl	50 µl

The plate was covered with an adhesive strip and incubated on a plate shaker, approximately 200 rpm for 1 h at room temperature. Subsequently, wells were aspirated and washed 5 times with wash buffer and the plate was tap firmly against absorbance paper to ensure that it is dry. The tipping off/ wash step was repeated. Then, 200 µl of Ellman's Reagent was added to each well and 5 µl of tracer to the TA well, and incubated again for 60-90 min with the plate covered. Finally, the optical density readings were recorded at 412nm using a microplate reader.

%B/Bo calculation:

(1) Average absorbance of NSB

(2) Average absorbance of Bo

(2) – (1) = Corrected Bo

To calculate the %B/Bo:

$$\frac{\text{Absorbance of each sample/kit standard} - (1)}{\text{Corrected Bo}} \times 100$$

To determine the PGE_2 concentrations, %B/Bo (%Bound/maximum Bound) was calculated. A standard curve (%B/Bo of kit standards against PGE_2 concentrations) was plotted and the equation obtained was used to calculate the PGE_2 of each sample, as shown in *Figure 2.2*.

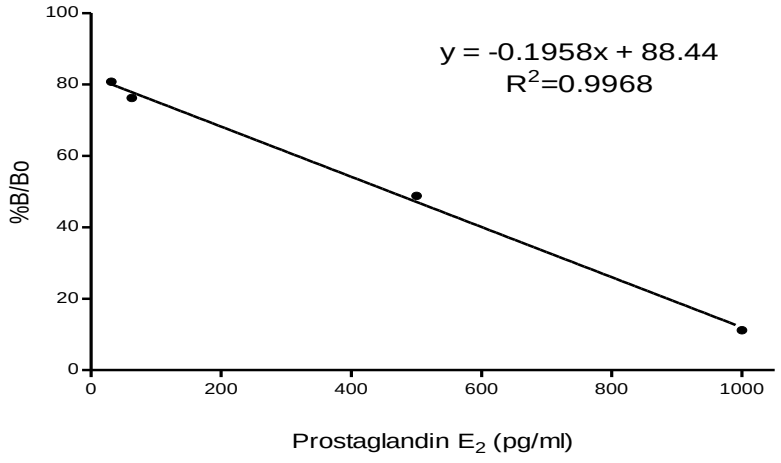


Figure 2.2: A standard curve to determine PGE_2 concentrations.

2.12 Statistical analysis

Data were analysed by 1-factor or repeated measures 2-factor ANOVA with Bonferroni's *post hoc* test. Except where otherwise indicated, all data are presented as the mean ± standard error of mean (SEM). Unpaired Student's *t-test* was used to compare two mean values. All tests were two-tailed and the significance level was set at $p<0.05$. Data analysis and graphs were plotted using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA.

CHAPTER THREE:

EFFECTS OF OXIDANTS AND ACROLEIN ON ENDOMETRIOTIC CELL GROWTH

3.1 Introduction

The role of ROS in female reproductive disease is becoming increasingly apparent. Other than endometriosis, ROS have been implicated in many diseases such as polycystic ovarian disease, preeclampsia and intrauterine growth retardation (Gupta *et al*., 2006). Oxidative stress, which results from a serious imbalance between reactive oxygen species (ROS) and antioxidant defences of the target cell (Halliwell, 2007), can modify intercellular communication, protein kinase activity, membrane structure and function, and gene expression, all of which can modulate cell growth (Klaunig *et al.*, 1998). In addition, upon exposure to ROS, many types of cells show a wide range of responses that include increased proliferation, prevention of cell division, senescence, or necrosis/apoptosis. These all depend on various parameters such as cell surface receptors, signal transduction mechanisms and antioxidant levels (Halliwell, 2007). Although reported studies on the association between oxidative stress and endometriosis have been inconsistent, there is considerable evidence that oxidative stress is a consequence of the inflammatory reaction that occurs in endometriosis (Seeber *et al.*, 2010). Peritoneal fluid and serum are among the biological fluids that have been studied to determine the association between levels of ROS and endometriosis.

3.1.1 ROS in peritoneal fluid

3.1.1.1 Evidence for elevated ROS in peritoneal fluid

Given that endometriotic tissue is surrounded by peritoneal fluid, it is speculated that substance(s) present in the fluid may affect the growth of endometriotic cells. Peritoneal fluid has been shown to contain a plethora of substances, including cytokines, growth factors, prostaglandins and steroid hormones such as estrogen and progesterone (Ramey & Archer, 1993) and also various free-floating cells

including macrophages (Haney *et al.*, 1981). Thus, peritoneal fluid is proposed as a major factor controlling the peritoneal microenvironment that influences the development and progression of endometriosis (Agarwal *et al.*, 2005). Increased levels of ROS was found in the peritoneal fluid of women with endometriosis (Wang *et al.*, 1997). Mounting experimental and clinical evidence indicates that this presence of ROS increases the growth and adhesion of endometrial cells in the peritoneal cavity (Jackson *et al*., 2005). The increased levels of ROS in the peritoneal fluid of women with endometriosis was further supported by the findings of a significant low levels of vitamin E in the peritoneal fluid of women with endometriosis as compared to normal women (Murphy *et al.*, 1998), which proposed antioxidants in peritoneal fluid are consumed during oxidation reactions.

Previous studies found an increase oxidation of low-density lipoprotein in patients with endometriosis and oxidation modification of these molecules involves peroxidation of the lipid component that results in aldehydes release including malondialdehyde (Murphy *et al.*, 1998). However, there was no significant difference in the levels of malondialdehyde in peritoneal fluid comparing women with moderate to severe endometriosis, women with minimal-to-mild endometriosis and women without endometriosis (Arumugam & Dip, 1995).

Although multiple studies have shown an association between elevated ROS and endometriosis, some researchers have found no significant difference in the ROS levels between the peritoneal fluid of women with endometriosis and women underwent tubal ligation (control) (Ho *et al.*, 1997; Bedaiwy *et al.*, 2002).

3.1.1.2 Possible causes of ROS production in peritoneal cavity

Endometriosis is associated with a general inflammatory response in the peritoneal cavity, and the purpose of this inflammatory response is to clear the ectopic cells

and tissue from the cavity (Lambrinoudaki *et al.*, 2009). Multiple inflammatory cells including eosinophils, neutrophils and macrophages are attracted to the sites of inflammation and they are all capable of generating ROS. In support of this, the peritoneal fluid of women with endometriosis has been shown to have an increased number, concentration and activation of macrophages (Kyama *et al.*, 2008) that generates the production of ROS including H_2O_2, superoxide and hydroxyl radicals upon its activation (Zeller *et al.*, 1987; Mier-Cabrera *et al.*, 2009).

ROS may also results from metabolic byproducts of erythrocytes and apoptotic endometrial tissue accumulated in the endometrial implants. This may alter morphologic and functional properties of endothelial cells by increasing permeability and adhesion molecule expression, thus enhancing inflammatory process (Lambrinoudaki *et al*., 2009). Cell debris transplanted into the peritoneal cavity by menstrual reflux has a potential to elevate ROS in the peritoneal cavity (Murphy *et al.*, 1998). Possibly, the presence of all the elements that have been mentioned disrupt the balance between ROS and antioxidants in the peritoneal fluid that lead to elevated ROS and increased endometriotic cell proliferation (Murphy *et al.*, 1998).

The presence of iron in the peritoneal cavity has been reported to cause an increased in ROS in the peritoneal fluid of women with endometriosis as iron is a potent generator of ROS. Pelvic iron deposition is considered to be a typical feature of endometriosis (Van Langendonckt *et al.*, 2002b). This iron could originate from the lysis of erythrocytes carried into the pelvic cavity by retrograde menstruation (Arumugam, 1994) and also the bleeding from the endometriotic lesions (Van Langendonckt *et al.*, 2002c). A significant high levels of iron and ferritin, a major iron-storing protein, was determined in the peritoneal fluid of women with endometriosis when compared to normal women, which correlated with the severity of disease (Van Langendonckt *et al.*, 2002b).

3.1.2 ROS in systemic circulation

Although accumulated data have shown that ROS are increased locally at sites of endometriotic implants, the disease has also been associated with increased in systemic circulation of ROS. An increase in serum autoantibody titres to oxidised low-density lipoproteins (Ox-LDL), malondialdehyde-modified LDL and lipid peroxide-modified serum albumin was demonstrated in women with endometriosis compared to normal women (Shanti *et al.*, 1999).

The role of systemic ROS was also determined by measuring the markers of oxidative stress in women with and without endometriosis (controls), which include heat shock protein 70 (HSP70), HSP70b', thioredoxin (TRX) and ischemia-modified albumin (IMA) (Lambrinoudaki *et al.*, 2009). However, only HSP70b' was significantly higher in women with endometriosis as compared to controls. Heat shock proteins are intracellular proteins that induced to protect cells from different types of insults such as inflammation and infection. These findings indicate that the elevated ROS may not be confined to the peritoneal cavity but may extend to the circulation.

The elevated ROS in systemic circulation was further supported by a separate study that demonstrated antioxidants, vitamin C and E lower the oxidative stress markers including malondialdehyde (MDA) and lipid hydroperoxides (LOOHs) in endometriosis patients (Mier-Cabrera *et al.*, 2009). In that study, the endometriotic patients were received either placebo or both vitamin C and E for 6 months. Interestingly, comparing to the group that received placebo, the group that received both vitamins had significantly lower levels of MDA in the fourth month and LOOHs in the sixth month. Therefore, these markers were likely to be at similar levels at the early stage of the study, but later decreased due to the effects of antioxidants.

Unfortunately, little experimental and clinical data exists to support the role of systemic ROS in the pathogenesis of endometriosis. Therefore, comparing the two biospecimens (peritoneal fluid and serum), peritoneal fluid has been suggested to be more reliable as the results provide a more localized measure related to endometriosis, whereas the measured markers of oxidative stress in serum, may represent oxidative stress due to other causes in addition to endometriosis (Jackson *et al.*, 2005).

3.1.3 Previous work establishing oxidants as playing a role in endometriotic cell proliferation

ROS can be produced both endogenously and exogenously. Endogenous ROS may arise from normal cellular metabolism and oxidative phosphorylation whereas exogenous sources include drugs, hormones and chemicals (Klaunig *et al.*, 1998). The production of ROS was determined in stromal and epithelial endometriotic cells from ovarian endometriomas (Ngo *et al.*, 2009). In that study, superoxide anion ($O_2^{\cdot-}$) and H_2O_2 were measured by spectrofluorimetry following the reaction with dihydroethidium and dichlorodihydrofluoresce diacetate respectively. Endometriotic cells displayed higher endogenous in ROS production as compared to eutopic endometrium in 14 patients with endometriosis. The proliferative capacity of endometriotic cells was also measured using the thymidine incorporation. The results showed an increased in the proliferation rate by 50% in stromal endometriotic cells compared with stromal endometrium (control) and 65% in epithelial endometriotic cells as compared with epithelial endometrium (control).

An *in vitro* study using the primary endometrial stromal cells from women with and without endometriosis demonstrated hydrogen peroxide (H_2O_2) at low concentration (1 μM) significantly induced cell proliferation in samples obtained from women with endometriosis (Foyouzi *et al.*, 2004). In that study, cells were

exposed to H_2O_2 (100 nM–10 µM) for 48 h and cell viability was measured by MTT assay. The proliferative effects of 1 µM H_2O_2 at 48 h was later confirmed using the same method in human immortalized endometrial stromal cell line, also known as Yale Human Endometrial Stromal or YHES cell line (Wu & Guo, 2006).

The effects of xanthine oxidase at (3 µU/mL-1000 µU/mL) were also determined in primary endometrial stromal cells from women with and without endometriosis (Foyouzi *et al.*, 2004). Xanthine oxidase at 3 µU/mL-30 µU/mL significantly induced cell proliferation in cells that obtained from women with endometriosis as compared to normal women. An increased expression of xanthine oxidase, was also demonstrated in the endometrium of women with endometriosis as compared to normal women (Ota *et al.*, 2001). Xanthine oxidase catalyses the hydroxylation of hypoxanthine to xanthine and then further catalyses the oxidation of xanthine to uric acid. In this process, it generates superoxide and H_2O_2.

As mentioned earlier, increased levels of iron has been shown in endometriosis. Iron is important for cell proliferation as it catalyses the reactions involved in oxygen sensing, energy metabolism, respiration, folate metabolism and DNA synthesis (Van Langendonckt *et al.*, 2002a). Previous studies determined the effects of human menstrual endometrium in mice by injecting intraperitoneally, either human menstrual endometrium alone (controls) or with erythrocytes or desferrioxamine (DFO), an iron chelator (Defrere *et al.*, 2006). A significant increase in iron levels was noted in endometriotic lesions, peritoneal macrophages and peritoneal fluid in mice receiving human menstrual endometrium with erythrocytes, but not in mice receiving DFO. The cell proliferative activity was also significantly higher in the presence of erythroctyes, which is known to have high levels of iron. Thus, it is proposed that iron also has a role in endometriotic cell proliferation.

3.1.4 Chapter Aims

The work described in this chapter was thus undertaken to determine whether oxidants and the lipid peroxidation product acrolein lead to cell proliferation or cell death in an endometriotic cell line.

The specific objectives are:

i) To evaluate the effects of oxidants and acrolein on endometriotic cell proliferation. The oxidants used are hydrogen peroxide (H_2O_2) and menadione, whereas acrolein was chosen as a product of lipid peroxidation.

ii) To determine the concentrations of the above substances that lead to cell death.

3.2 Materials and Methods

Immortalized human endometriotic epithelial cells (12-z) were used in this study. Further information about this cell line has been described in Chapter 2 (2.1.1). Cells were treated with H_2O_2, menadione, or acrolein and cell viability was assessed by MTT and LDH assays that have been described in Chapter 2 (section 2.2.4.1 and 2.2.4.2 respectively).

3.3 Results

3.3.1 Effects of oxidants and acrolein on cell growth and viability of 12-z cells.

12-z endometriotic cells were used as a model to investigate cell proliferation. Different types of *in vitro* cytotoxicity assays have been used to detect cytotoxicity or cell viability following exposure to toxic substances, which include MTT, LDH, neutral red and protein assays (Fotakis & Timbrell, 2006). These have demonstrated that the MTT assay was one of the most sensitive cell viability assays. In the present study, MTT and LDH assays were used to determine the viability of cells. The MTT assay is mainly based on the enzymatic conversion of MTT in the mitochondria whereas the LDH leakage assay is based on the release of the enzyme into the culture medium after cell membrane damage.

3.3.1.1 Effects of H_2O_2 on cell growth

H_2O_2 has been used widely as an oxidant in *in vitro* studies in many types of cells. It causes direct oxidative injury to cells whereby it acts in a concentration-dependent manner to induce cell death (Bae *et al.*, 2007). However, at lower concentration it causes mild oxidative stress, which has been reported previously to increase endometrial stromal cell proliferation from women with endometriosis (Foyouzi *et al*., 2004).

In order to evaluate the effects of H_2O_2 on cell survival, 12-z cells were exposed to H_2O_2 at concentrations of 0.5 µM – 500 µM for 24 h and 48 h and cell growth determined using the MTT assay. This assay gives an indication of the metabolic activity of a population of cells. As shown in *Figure 3.1*, treatment of 12-z cells with H_2O_2 significantly induced cell proliferation at 1 µM at 48 h of culture where cell viability was 116% of control. In contrast, H_2O_2 at higher concentrations inhibited cell growth especially after 48 h of culture.

3.3.1.2 Effects of menadione on cell growth

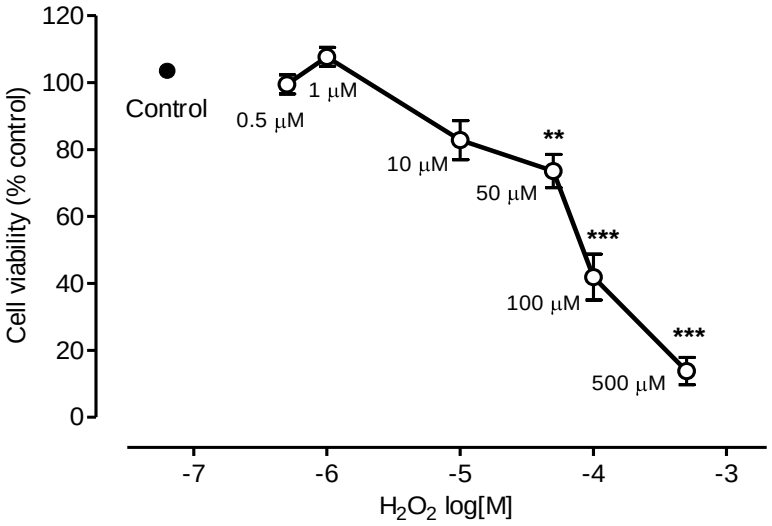
Menadione (2-Methyl-1, 4-naphthoquinone), is a naphthoquinone. In the presence of molecular oxygen, menadione generates the superoxide anion radical ($O_2^{\cdot -}$), leading to the formation of H_2O_2 and other ROS. Similar to H_2O_2, the direct effect of menadione on endometriotic cells has not been examined previously. However, menadione has been shown to have toxic effects on bovine embryo (Moss *et al.*, 2009). To determine the effects of menadione on cell growth, 12-z cells were treated with menadione at concentrations of 10 µM – 35 µM for 24 h and 48 h and cell viability measured using the MTT assay. As shown in *Figure 3.2*, menadione at 20 µM significantly induced cell proliferation after 48 h of culture, increasing cell viability to 117% of control. However, higher concentrations of menadione inhibited cell growth significantly especially at 48 h.

3.3.1.3 Effects of acrolein on cell growth

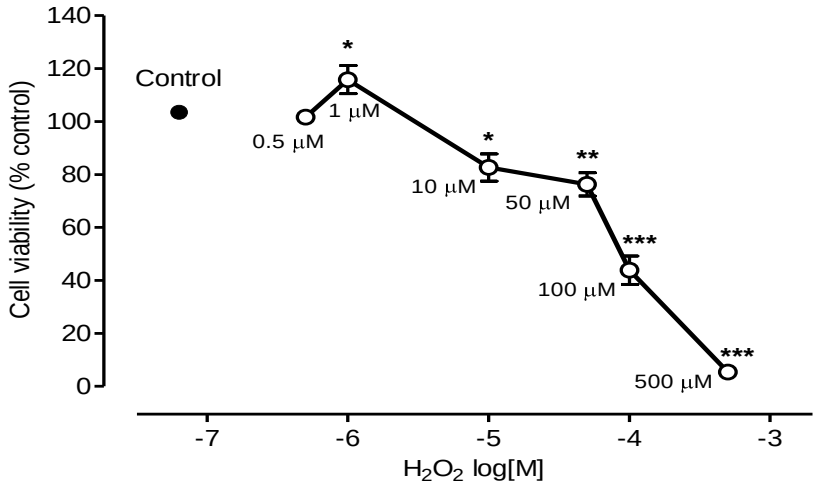
Acrolein (α, β-unsaturated aldehyde), is a lipid peroxidation product. No previous study has looked at the direct effect of acrolein on endometriotic cells. Acrolein has been reported to be one of the most reactive aldehydes. To examine the effects of acrolein on cell survival, 12-z cells were exposed to acrolein at concentrations of 10 µM – 100 µM for 24 h and 48 h and viability measured using MTT assays. As shown in *Figure 3.3*, acrolein significantly induced cell proliferation at 20 µM at 48 h of culture with the percentage of cell viability was 122% of control. Similar to the oxidants, cell growth was inhibited when exposed to higher concentrations of acrolein.

Figure 3.1: Effects of H_2O_2 on cell growth

a)



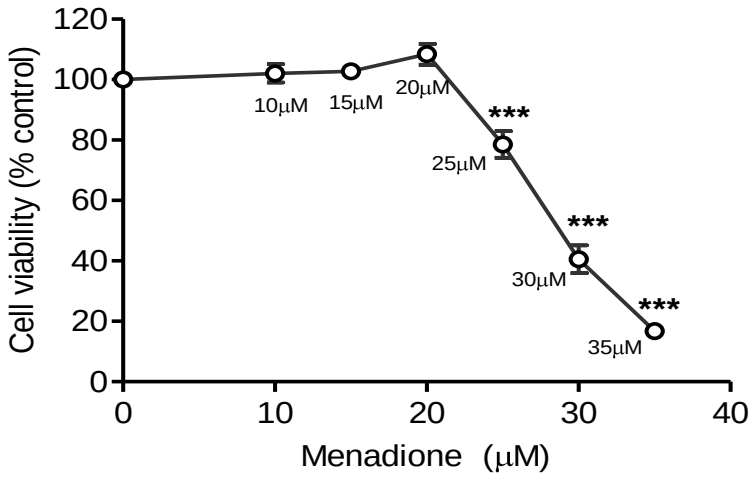
b)



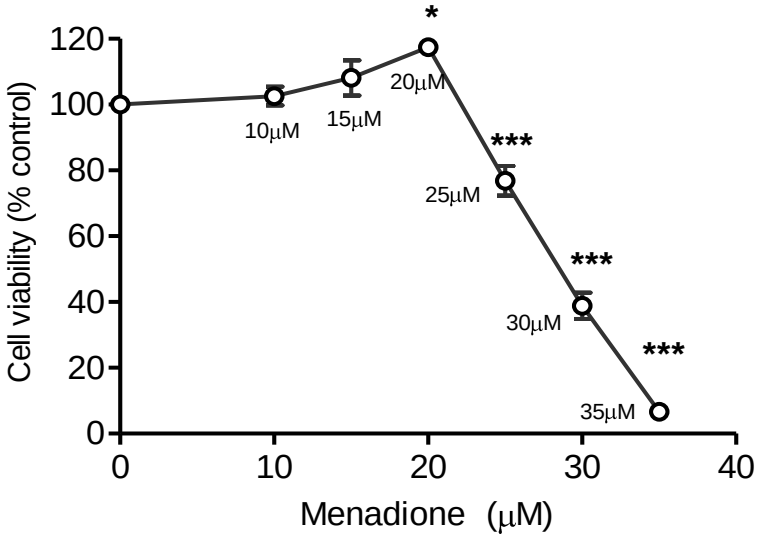
12-z cells were treated with H_2O_2 at concentrations shown for a) 24 h and b) 48 h. Cell viability was measured using the MTT assay. Values represent mean ± SEM (n=6) and are expressed as percentage of control (untreated) cells.
* p<0.05, ** p<0.01 & *** p<0.001 vs. Control.

Figure 3.2: Effects of menadione on cell growth

a)



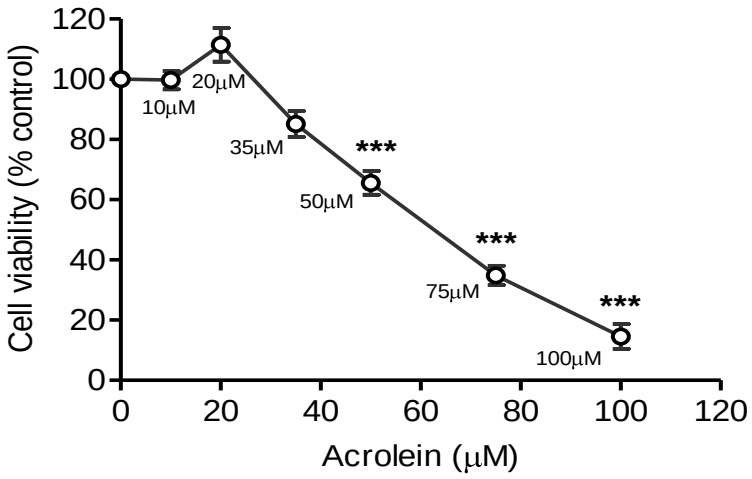
b)



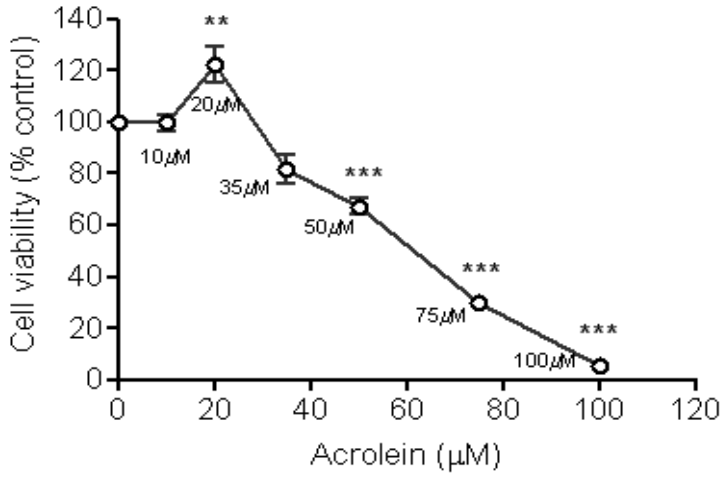
12-z cells were treated with menadione at concentrations shown for a) 24 h and b) 48 h. Cell viability was measured using the MTT assay. Values represent mean ± SEM (n=6) and are expressed as percentage of control (untreated) cells.
* $p<0.05$, ** $p<0.01$ & *** $p<0.001$ vs. Control

Figure 3.3: Effects of acrolein on cell growth

a)



b)



12-z cells were treated with acrolein at concentrations shown for a) 24 h and b) 48 h. Cell viability was measured using the MTT assay. Values represent mean ± SEM (n=6) and are expressed as percentage of control (untreated) cells.
* $p<0.05$, ** $p<0.01$ & *** $p<0.001$ vs. Control.

3.3.2 Effects of oxidants and acrolein on cell membrane integrity

The lactate dehydrogenase (LDH) leakage/release assay was used to determine cell membrane integrity, which is based on the measurement of LDH activity in the extracellular medium (Fotakis & Timbrell, 2006). To evaluate *in vitro* cytotoxicity of oxidants and acrolein, cells were exposed to H_2O_2 (0.5 µM – 500 µM), menadione (10 µM – 35 µM) and acrolein (10 µM – 100 µM) for 24 h and 48 h. LDH leakage was measured using Tox1 assay kit (Sigma). The percentage of LDH leakage was calculated as ratio between LDH activity in culture medium and that of the whole cell content.

3.3.2.1 Effects of H_2O_2 on cell membrane integrity

As shown in *Figure 3.4*, there was no significant difference in LDH leakage from 12-z cells treated with low concentrations of H_2O_2 at 24 h or 48 h. However, in the presence of H_2O_2 at moderate to high concentrations, a significant increase in LDH leakage was observed at 24 h (100 µM – 500 µM) and 48 h (50 µM – 500 µM).

3.3.2.2 Effects of menadione on cell membrane integrity

Similar to the effects of H_2O_2, there was no significant difference in LDH leakage from 12-z cells treated with low concentrations of menadione at 24 h or 48 h. In contrast, at moderate to high concentrations, an increase in LDH leakage was observed especially at 48 h of treatment, as shown in *Figure 3.5*.

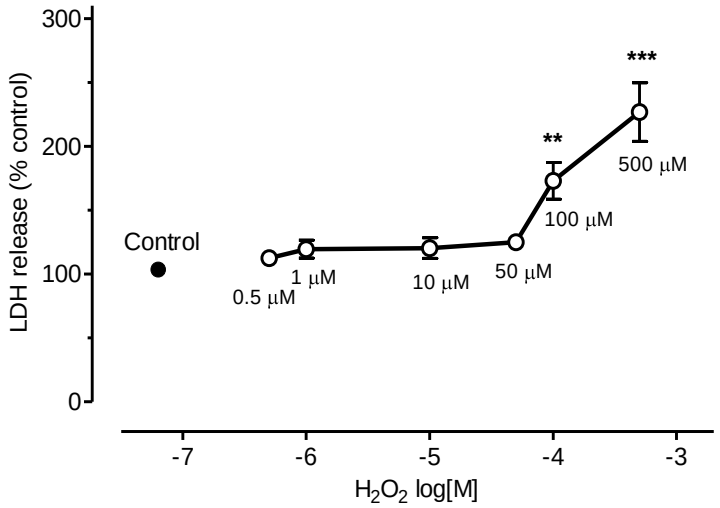
3.3.2.3 Effects of acrolein on cell membrane integrity

As shown in *Figure 3.6*, the LDH leakage revealed no significant different in LDH leakage at low concentrations of acrolein as compared to control. However, the LDH leakage was significantly increase when cells were treated with higher concentrations of acrolein where there was membrane ruptured at 75 µM – 100 µM after 24 h and at 48 h, cells that were treated with 50 µM – 100 µM acrolein had significant increase in LDH leakage as compared to control.

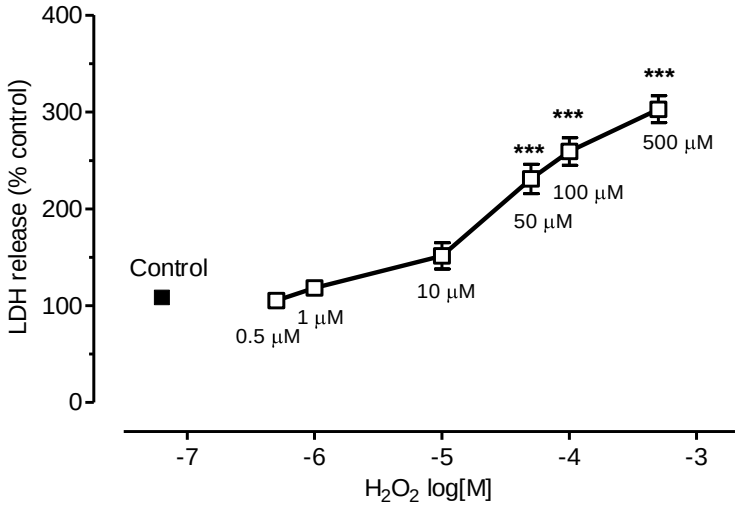
Therefore, all the three compounds are toxic to 12-z cells at moderate to high concentrations, which is consistent with MTT assay results. The release of intracellular LDH into the culture medium is an indicator of irreversible cell death (necrosis) due to cell membrane damage.

Figure 3.4: Effects of H_2O_2 on LDH release

a)



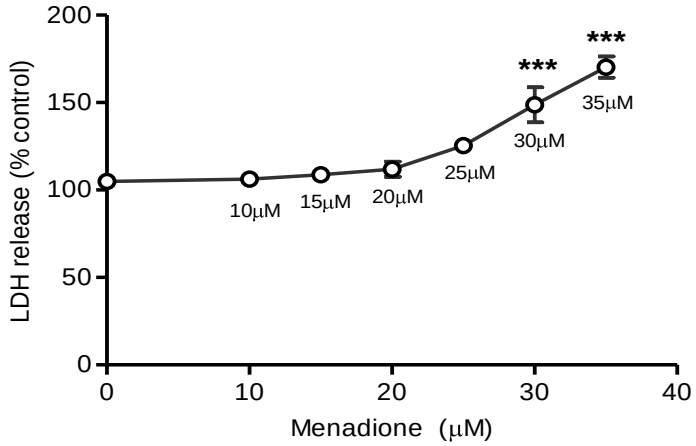
b)



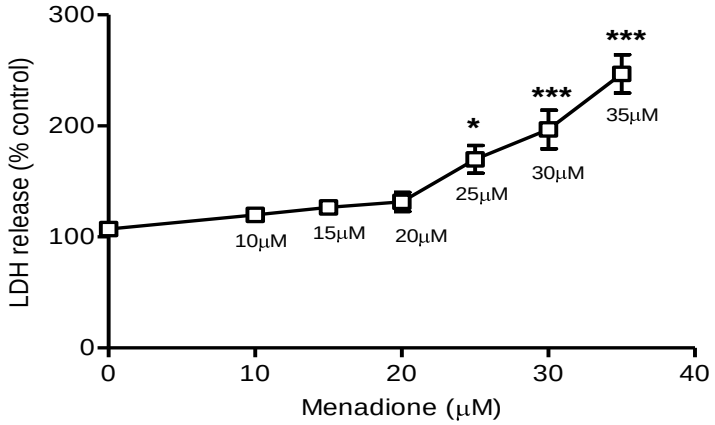
12-z cells were treated with H_2O_2 at the concentrations shown for a) 24 h and b) 48 h. The level of LDH leakage was measured by Tox1 assay kit (Sigma). Values represent mean $\pm$ SEM (n=6) and % LDH leakage was calculated as ratio between LDH activity in culture medium and that of the whole cell content.
*p<0.05, ** p<0.01 & *** p<0.001 vs. Control.

Figure 3.5: Effects of menadione on LDH release

a)



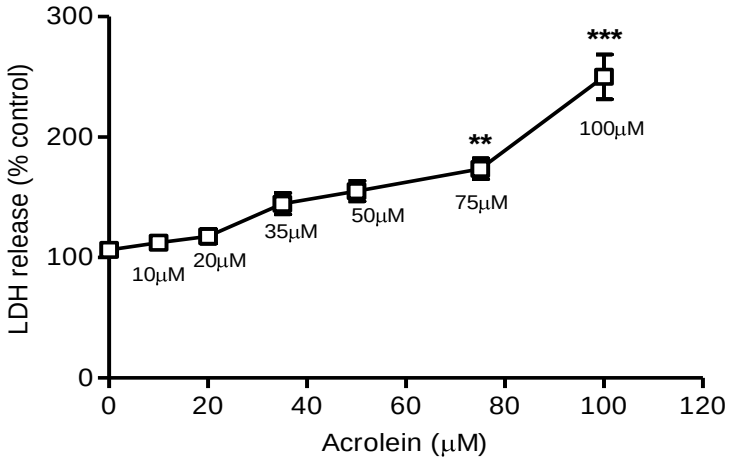
b)



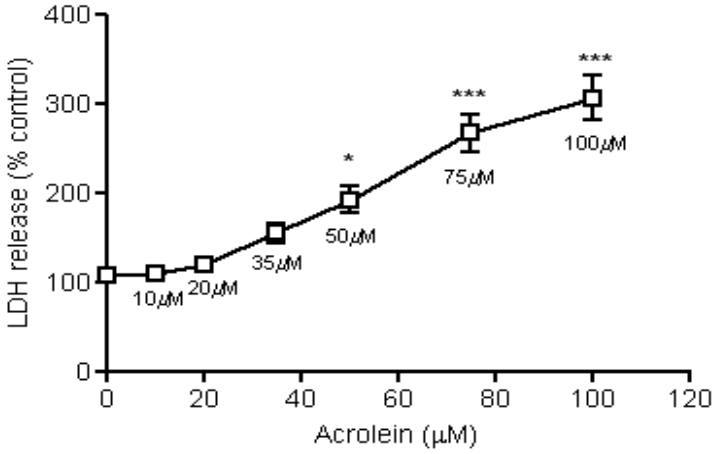
12-z cells were treated with menadione at the concentrations shown for a) 24 h and b) 48 h. The level of LDH leakage was measured by Tox1 assay kit (Sigma). Values represent mean ± SEM (n=6) and % LDH leakage was calculated as ratio between LDH activity in culture medium and that of the whole cell content.
*p<0.05, ** p<0.01 & *** p<0.001 vs. Control.

Figure 3.6: Effects of acrolein on LDH release

a)



b)



12-z cells were treated with acrolein at the concentrations shown for a) 24 h and b) 48 h. The level of LDH leakage was measured by Tox1 assay kit (Sigma). Values represent mean ± SEM (n=6) and % LDH leakage was calculated as ratio between LDH activity in culture medium and that of the whole cell content.
*p<0.05, ** p<0.01 & *** p<0.001 vs. Control.

3.4 Discussion

Oxidative stress is well known to precipitate the range of pathologies that affects reproductive function. Factors that cause or the effects of oxidative stress can modify cell functions, impair cell survival or both (Agarwal *et al.*, 2003). The results of this chapter provide evidence that oxidants and acrolein induce endometriotic cell proliferation at low concentrations but cause cell death at moderate to high concentrations.

3.4.1 Oxidants and acrolein at low concentrations lead to cell proliferation

The results revealed a significant increased in cell proliferation in the presence of H_2O_2, menadione and acrolein at low concentrations after 48 h of treatment. These results supports an earlier *in vitro* study using the primary endometrial stromal cells (Foyouzi *et al.*, 2004) and YHES cell line (Wu & Guo, 2006), which is also consistent with more general studies that show low level of oxidants promotes cell proliferation (Halliwell, 2007). Our study is the first to demonstrate a proliferative effect with H_2O_2, menadione and acrolein in endometriotic cells.

3.4.2 Oxidants and acrolein at moderate to high concentrations lead to cell death

In contrast, at higher concentrations, H_2O_2, menadione and acrolein caused a loss of viability. The toxic effects of oxidative stress on cells depend upon the intensity of the stress and/or the cell type (Dragin *et al.*, 2006). The cytotoxicity of H_2O_2 at high concentration (100 µM) has also been demonstrated previously using the primary endometrial stromal cells (Foyouzi *et al*., 2004), but this is the first time that menadione has been investigated. Accumulation of ROS may cause a deleterious process that can be an important mediator of damage to cell structure, including

lipids and membranes, proteins and DNA that inhibit their normal function (Valko *et al.*, 2007). The effects of elevated ROS on cells have been described in Chapter 1.

We have also shown that acrolein causes cell death at high concentrations. The sources of acrolein can be grouped into dietary, endogenous and environmental sources (Stevens & Maier, 2008). Endogenously, acrolein is a toxic by-products of lipid peroxidation resulting from oxidative stress and has been implicated in many diseases including cardiac, pulmonary and neurodegenerative diseases (Roy *et al.*, 2009). Several mechanisms by which acrolein results in cellular toxicities have been described. At concentration of 100 µM, acrolein has been shown to cause neuronal cell death (Liu-Snyder *et al.*, 2006). Acrolein has also been reported to alter Ca^{2+} homeostasis and disruption of mitochondria oxidative phosphorylation leading to cell death, which is primarily by necrosis rather than apoptosis (Hansen *et al.*, 2007). In addition, acrolein induces oxidative modification of proteins which either results in dysregulation of specific signaling pathways or protein inactivation (Uchida *et al*., 1998). However, there is no available data showing that acrolein is involved in cell proliferation.

Cytotoxicity induced by the compounds used in this study was further assessed by LDH leakage into the medium. The results of LDH assay were consistent with the MTT assay whereby when cells were exposed to high concentrations of these substances, the percentage of LDH leakage was significantly higher than control, which indicate at high concentrations there is increased in cell death. Therefore, rupture of the cell membrane occurs when cells were exposed to high concentrations of oxidants especially after 48 h.

Therefore, the toxic effects of oxidative stress or its products on cells depend upon the intensity of the stress. It is acknowledged that at least some form of cell damage are initiated by compounds added into the culture media, the extracellular environment, although the degree of cell permeability by any compounds is not

known (Yoshida *et al*., 2009). A biphasic dose-response has been suggested to describe effect of oxidative stress on endometriotic cells as at low level of oxidative stress it caused significant in cell proliferation whereas at higher level it inhibited cell growth (Foyouzi *et al*., 2004). ROS has been linked to numerous biological processes when they are produced at different levels such as at high levels they may exert damaging effects (Al-Gubory *et al.*, 2010). This is supported by a previous study using hepatoma cells that low ROS concentrations have been shown to increase the antioxidant enzyme activity that promote cell proliferation whereas high concentrations of ROS induced apoptosis or necrosis (Dragin *et al.*, 2006).

3.4.3 Levels of ROS that occur in endometriosis

Previous studies have reported the high reactivity and relative instability of ROS make them extremely difficult to detect or measure *in vivo* (Thannickal & Fanburg, 2000). Therefore, most of the researchers measured ROS and free radical generation by indirect measurement of various end products resulting from the interaction of ROS with cellular components such as lipids, protein or DNA. Numerous assays have been used to measure ROS either direct methods; such as electron paramagnetic resonance spectroscopy for free radicals, and indirect methods such as flow cytometry and chemiluminescence assay, one of the most commonly methods to measure ROS levels (Kobayashi *et al.*, 2001).

Levels of ROS were determined in peritoneal fluid from patients with endometriosis and the control group (patient underwent tubal ligation) by chemiluminescence assay (Agarwal *et al.*, 2003). In that assay, a luminol-mediated chemiluminescence signal probe was used, which can measure H_2O_2, $O_2^{\cdot -}$ and OH^- levels, although it cannot distinguish these oxidants from one another. The ROS production was expressed as counted photons per minute (cpm)x10^6. The levels of ROS were not statistically significant between the two groups with the values less than 2x10^6 cpm.

In addition, they have also demonstrated the ROS levels were stable throughout the menstrual cycle in both of the groups and for the endometriosis group, the levels did not correlate with the stage of the disease.

Ngo and colleagues have measured levels of superoxide anion ($O_2^{\cdot-}$) and H_2O_2 in stromal and epithelial endometriotic cells using spectrofluorimetry (Ngo *et al.*, 2009). They have found a significant increase in H_2O_2 production with 2.5-fold higher in stromal endometriotic cells and 7.75-fold higher in epithelial endometriotic cells when compared to stromal control cells and epithelial control cells respectively. However, the different levels between the stromal and epithelial endometriotic cells is poorly understood. Thus, it is obvious that endometriotic cells display an elevated ROS that may lead to oxidative stress.

Van Langendonckt and colleagues measured concentrations of free iron, a strong pro-oxidant in the peritoneal fluid of women with and without endometriosis at different menstrual cycle phase (Van Langendonckt *et al.*, 2002b). These authors demonstrated that during the secretory phase, iron concentrations in the peritoneal fluid from women with endometriosis were significantly higher than controls with the values were 80 mg/ml and 60 mg/ml respectively. However, there was no significant difference in the iron levels between the two groups at proliferative phase with the values were approximately 60 mg/ml. These authors have suggested ovulation that occur at the end of the proliferative phase, may release iron in the peritoneal fluid, which was detected during the secretory phase. One of the reasons causing the discrepancy in the results was proposed due to the oxidative stress may occur locally, at the site of bleeding rather than involving the total peritoneal fluid (Van Langendonckt *et al.*, 2002c).

Although an oxidative marker HSP70b' was found significantly higher in women with endometriosis as compared to normal women, the values were not markedly

different; 0.178 ng/ml and 0.135 ng/ml respectively (Lambrinoudaki *et al.*, 2009). HSP70b' is the major representative of heat shock protein. Based on these published data, it is appear that the levels of ROS observed in endometriosis are likely to be relatively low, and would therefore be expected to induce cell proliferation rather than cell death.

3.4.4 Mechanism of cell proliferation by low levels of ROS

Endometriosis is well accepted as a chronic inflammatory disease with ROS as one of the pro-inflammatory mediators that modulate cell proliferation (Ngo *et al.*, 2009). Previous studies have proposed ROS as a second messenger in the mechanism of normal cell proliferation through the activation of growth-related signaling pathways including mitogen-activated protein kinase ERK1/2 (McCubrey *et al.*, 2006). It is interesting to note that Ngo and colleagues have also demonstrated the involvement of this pathway in the mechanism of cell proliferation in endometriotic cells using the Western blotting (Ngo *et al.*, 2009). As expected, the phosphorylation of ERK was almost undetectable in control cells. In contrast, the phosphorylation of ERK was significantly upregulated in both stromal and epithelial endometriotic cells as compared to stromal endometrial and epithelial endometrial cells respectively from normal women. In addition, these authors also demonstrated a significant increase in ERK phosphorylation in endometrial stromal cells and endometrial epithelial cells from women with endometriosis as compared to similar types of cells from normal women, despite no significant different in the levels of H_2O_2. They have proposed that although the H_2O_2 production was low, the ERK signalling pathway was still activated. Despite numerous published data regarding the association of ROS levels in women with endometriosis and its pathogenesis, the mechanism of cell proliferation in this disease was not clarified by most of the authors.

Although the mechanism of cell proliferation due to ROS exposure in endometriosis is poorly understood, roles of ROS in controlling tumour cell proliferation have been well reported (Valko *et al.*, 2006). These authors proposed ROS at high concentrations cause cell death or even necrosis while at low concentrations, it causes cell proliferation and enhanced cell survival. Low levels of ROS may alter gene expression through interaction and modification of genome DNA especially the growth factors (Frenkel, 1992). ROS is able to activate downstream transcription factors including nuclear factor erythroid 2-related factor 2 (NF-E2/rf2 or Nrf2), mitogen-activated protein (MAP) kinase/AP-1 and NF-κB pathways, which are involved in cell proliferation. As endometriotic cells share important similarities with neoplastic processes such as proneness to invasion, neoangiogenesis and distant spreading (Matalliotakis *et al.*, 2007), the correlation of ROS and cancer cell proliferation may suggest the same possible role for ROS in the regulation of cell proliferation in endometriosis. Low concentrations of H_2O_2 (0.2 nM-20 nM) induced cancer cell proliferation whereas H_2O_2 at higher concentrations (100 µM – 1000 µM) showed cytotoxicity was demonstrated previously using the human cervical carcinoma cell line HeLa-Ohio (HeLa) (Liu *et al.*, 2003).

In summary, the MTT assay indicates that low concentrations of oxidants and acrolein stimulate cell proliferation whereas at high levels they cause cell death, which later was confirmed by the LDH assay. The concentrations of test compounds that induced cell proliferation, will be used in subsequent experiments in this thesis. It has also to be determined whether other factors are involved in the mechanism of cell proliferation in endometriosis that include estradiol, steroid receptors, antioxidants, and the involvement of signaling pathway/s.

CHAPTER FOUR:

EFFECTS OF ESTRADIOL, OXIDANTS AND ACROLEIN ON ENDOMETRIOTIC CELL GROWTH

4.1 Introduction

4.1.1 Roles of estrogen biosynthesis and metabolising enzymes in endometriosis

Estrogen is essential for endometriotic cell growth (Zeitoun *et al.*, 1998; Izawa *et al.*, 2008), and if deprived of this hormone, endometriotic cell tends to regress (Nawathe *et al.*, 2008). The occurrence of the disease mainly during reproductive period of life can be explained on the basis of an increased estrogen stimulation (Vinatier *et al.*, 2001). Defective metabolism of estrogen plays an important role in disease development. Thus, this hormone seems to play an essential role in endometriosis. Aberrant expression of several estrogen biosynthesis and metabolising enzymes such as aromatase, 17β-HSD type 1 and type 2, AKR1C3 and COX-2 has been observed in endometriotic cells, which leads to high estrogen biosynthesis and low estrogen inactivation that subsequently results in further proliferation of endometriotic cells (Giudice & Kao, 2004). Previous studies of the difference in these enzymes expression in endometriotic cells, normal endometrium and endometrium from women with endometriosis have been mentioned earlier in Chapter 1 (section 1.1.4.1)

4.1.2 A *'Positive feedback loop'* for E2 production

The involvement of estrogen biosynthesis enzymes has lead to continuous estrogen production particularly estradiol (E2), which has been described in endometriotic stromal cells as a *'positive feedback loop'* (Zeitoun *et al.*, 1998). This loop has described androstenedione (A) from adrenal gland and/or ovary is converted to estrone (E1) by aromatase P450 (Zeitoun *et al.*, 1998; Bulun *et al.*, 2002a; Rizner, 2009). E1 is then reduced to E2 by 17β-HSD type 1 (Zeitoun *et al.*, 1998; Rizner, 2009). Subsequently, E2 either directly stimulates COX-2 or endometriotic cells may also induce inflammation in the peritoneal cavity that stimulate peritoneal macrophages to release cytokines, interleukin (IL)-1β and tumor necrosis factor

(TNF)-α (Tamura *et al.*, 2002b), which may also stimulates COX-2 that gives rise to the production of PGE_2 (Carli *et al.*, 2009). PGE_2 was known to be the most potent known inducer of aromatase activity in endometriotic stromal cells (Noble *et al.*, 1997). This establishes a *'positive feedback loop'* in favour of continuous E2 formation in endometriosis, as shown in *Figure 4.1*. In addition, a deficiency of 17β-HSD type 2 expression, impairs the inactivation of E2 to E1 (Bulun *et al.*, 2002a). This indicates that estrogen synthesis is occurring continuously (Kitawaki *et al.*, 1997). However, the mechanism of E2 production in endometriotic epithelial cells especially factors that may influence its expression, needs further investigations.

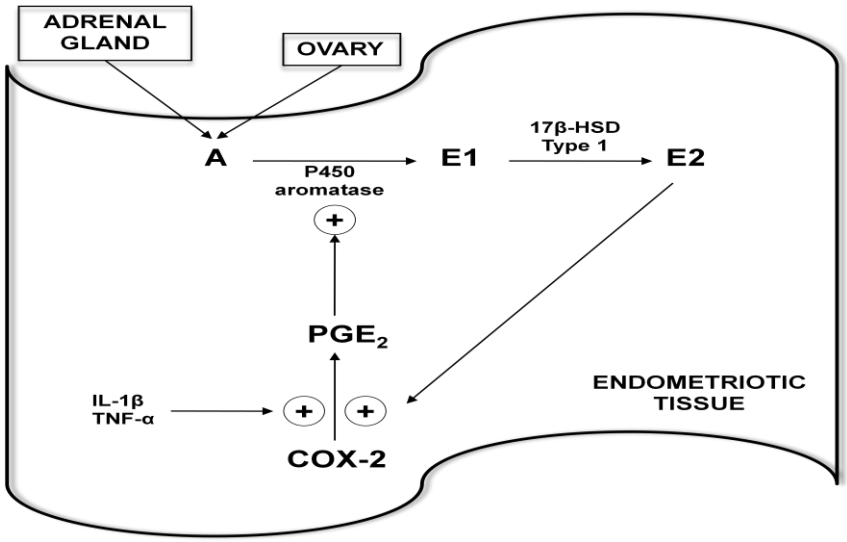


Figure 4.1: A *'Positive Feedback Loop'* for E2 production in endometriotic stromal cells. Androstenedione (A), the most important precursor of E2 arises from adrenal and ovary is converted to E1 by aromatase P450 and later reduced to E2 by 17β-HSD type 1. Endometriotic tissue can induce COX-2 through the formation of cytokines, IL-1β and TNF-α. E2 is also known to induce COX-2 expression. COX-2 increases the production of PGE_2, a potent stimulator of aromatase enzymes. The pathways lead to continuous local E2 production that promotes endometriotic cell growth and proliferation. Adapted from (Zeitoun *et al.*, 1998).
A: androstenedione, E1: estrone, E2: estradiol, COX-2: cyclooxygenase-2, PGE_2: prostaglandin E_2.

4.1.3 Estrogen receptors in endometriosis

Both of ER-α and ER-β are present in the endometrium of normal women and women with endometriosis (Matsuzaki *et al.*, 2000) and in endometriotic cells (Hudelist *et al.*, 2005). However to date, the exact ER subtypes that mediates the action of estrogen as well as its mechanism of action in endometriosis is still controversial. The different expressions and roles of ER-α and ER-β in endometriosis have been described in Chapter 1 (section 1.1.4.1).

4.1.4 Chapter Aims

With evidence implicating the involvement of estrogen biosynthesis enzymes in the mechanism of cell proliferation, it is essential to identify factors that influence cell growth and proliferation in endometriosis. The identification of factors that involved in the mechanism of endometriotic cell proliferation may lead to valuable therapeutic approaches for limiting the disease progression.

To achieve this aim, this study has set the following objectives:

1. To examine the effects of E2 on 12-z cells growth
2. To examine whether E2 has any effect e.g. synergistic or additive, with oxidants or acrolein in promoting 12-z cells growth
3. To determine whether E2 and/or oxidants or acrolein can regulate the expression of various genes in 12-z cells
   i) aromatase, COX-2, PGE_2, 17β-HSD type 1 and type 2, and AKR1C3
   ii) steroid receptors (ER-α and ER-β)
4. To determine the effects of E2 and/or oxidants or acrolein on aromatase activity in 12-z cells
5. To determine aromatase mRNA expression of samples from patients with ovarian endometriosis and patients with *Uterine myomatosus* as control.

4.2 Materials and methods

12-z cells were treated with oxidants, acrolein, E2 or combination of both E2 with oxidants or acrolein. Besides the cell line, samples from ovarian endometriosis and *Uterine myomatosus* were also studied.

All other materials and methods for this chapter are described in chapter 2.

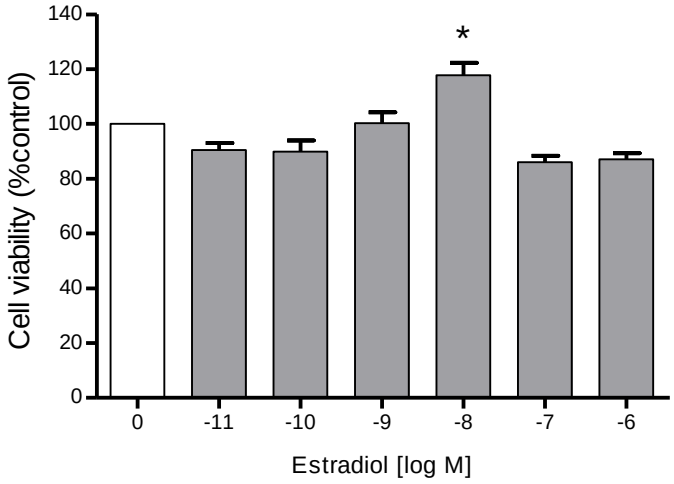
4.3 Results

In order to discover which factors had a significant influence on cell growth, 12-z cells were used and treated with E2, oxidants and acrolein. The results of previous chapter (Chapter 3) have demonstrated that 1 µM H_2O_2, 20 µM menadione and 20 µM acrolein significantly induced 12-z cells proliferation at 48 h.

4.3.1 Effects of E2 on cell growth and viability

In order to see which concentrations of E2 were effective, 12-z cells were plated in 96 well plates at cell densities of 5000 – 10 000 cells per well and allowed to attach for 24 h. Then, cells were treated with different concentrations of E2 (17β-estradiol) at 10^{-11} , 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7} and 10^{-6} M for 24 h. *Figure 4.2* demonstrates the effects of E2 on 12-z cells that cultured for 24 h as measured using the MTT assay. E2 at 10^{-8} M significantly induced cell proliferation where percentage of cell viability was 117% of control. This is the first cell viability study of the effects of E2 on the growth of endometriotic cells.

Figure 4.2: Effects of E2 on cell growth



12-z cells were treated with E2 at concentrations shown for 24 h. Cell viability was measured using the MTT assay. Values represent mean ± SEM (n=6) and are expressed as percentage of control (untreated) cells.
* $p<0.01$ vs. Control.

4.3.2 Effects of oxidants or acrolein and E2 on cell growth and viability

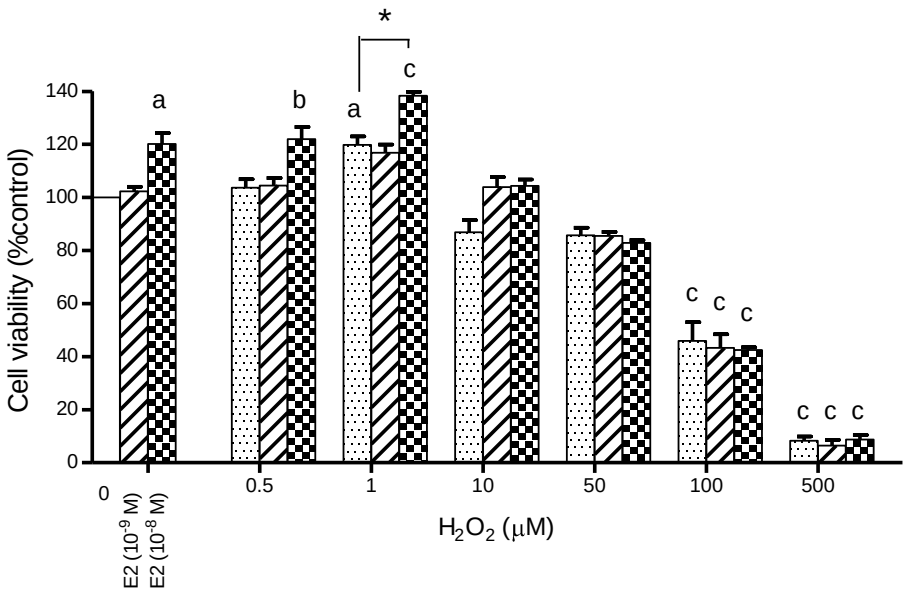
To determine whether oxidants or acrolein has additive or synergistic effects with E2 in promoting endometriotic cell growth, cells were pre-treated with E2 at 10^{-9} M (physiological concentration) and 10^{-8} M for 24 h followed by addition of oxidants or acrolein and cultured for another 48 h.

4.3.2.1 Effects of H_2O_2 and E2 on cell growth and viability

In this experiment, cells were pre-treated with E2 at 10^{-9} M (physiological concentration) or 10^{-8} M for 24 h followed by addition of 0.5 µM – 500 µM H_2O_2 and cultured for another 48 h. Untreated cells were used as control. As shown in *Figure 4.3*, similar to previous chapter (Chapter 3), 1 µM H_2O_2 significantly induced cell proliferation at 48 h with the percentage of cell viability was 120% of control (untreated). However, higher concentrations of H_2O_2 at 100 µM – 500 µM inhibited cell growth significantly.

E2 at 10^{-8} M significantly induced cell proliferation with the percentage of cell viability was 120%. However, when cells were exposed to E2 at physiological concentration, it did not show a significant increase in cell proliferation. When cells were pre-treated with E2 at 10^{-8} M, a significant increased in cell proliferation was observed when cells were treated with H_2O_2 at 0.5 µM or 1 µM where the percentage of cell viability was 122% and 138% of control respectively. The relative viability of cells that were treated with 1 µM H_2O_2 alone was significantly different when compared to cells that were pre-treated with E2 at 10^{-8} M followed by addition of 1 µM H_2O_2. However, 100 µM – 500 µM H_2O_2 inhibited cell growth significantly despite the presence of E2.

Figure 4.3: Effects of H_2O_2 and E2 on cell growth



12-z cells were pre-treated with or without E2 at 10^{-9} M or 10^{-8} M for 24 h followed by addition or without addition of H_2O_2 at concentrations shown for 48 h. Cell viability was measured using the MTT assay. Values represent mean ± SEM (n=6) and are expressed as percentage of control (untreated) cells.

[a] $p<0.05$, [b] $p<0.01$, [c] $p<0.001$ vs. Control.

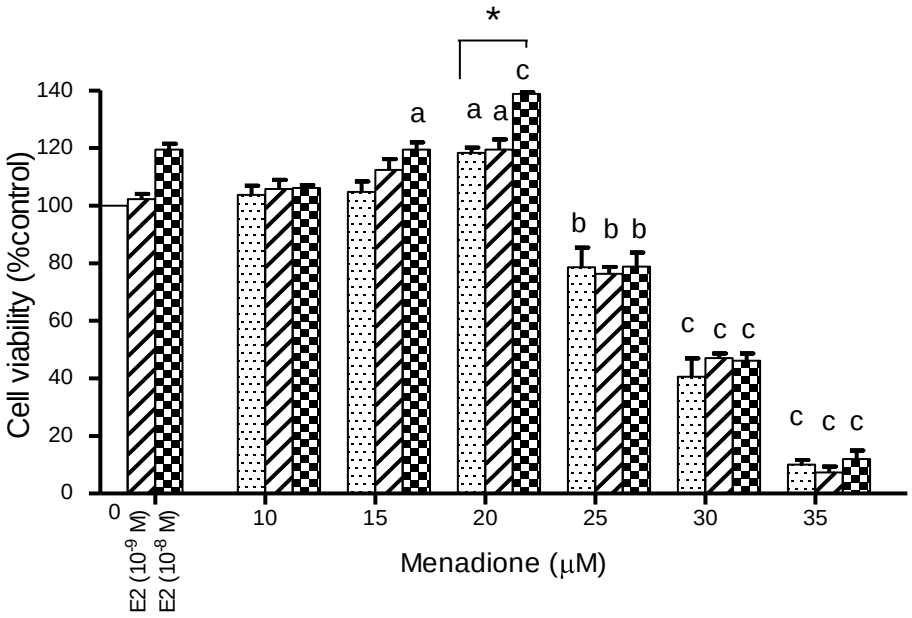
* $p<0.05$ H_2O_2 alone vs. H_2O_2 with 10^{-8} M E2

4.3.2.2 Effects of menadione and E2 on cell growth and viability

To test the effects of menadione in the presence of E2, cells were pre-treated with E2 at 10^{-9} M (physiological concentration) or 10^{-8} M for 24 h followed by addition of menadione at 10 µM – 35 µM and cultured for another 48 h. As demonstrated in *Figure 4.4*, a significant increase in cell proliferation was observed when cells were cultured with menadione at 20 µM for 48 h where the percentage of cell viabiliy was 118% of control. In contrast, 25 µM – 35 µM menadione inhibited cell growth significantly.

E2 at 10^{-8} M significantly induced cell proliferation where the percentage of cell viability was 119% of control. Although a significant increase in cell proliferation was observed when cells were pre-treated with 10^{-9} M followed by the addition of menadione at 20 µM, the percentage of cell viability was 119% of control, which is similar to when cells were treated with menadione alone at 20 µM. E2 at 10^{-8} M further increased cell proliferation in cells treated with menadione at 20 µM where the percentage of cell viability was 139% of control. Cell viability following treatment with 20 µM menadione alone was significantly different to that observed in cells that were pre-treated with E2 at 10^{-8} M followed by addition of 20 µM menadione. Menadione at 25 µM – 35 µM inhibited cell growth significantly despite the presence of E2.

Figure 4.4: Effects of menadione and E2 on cell growth



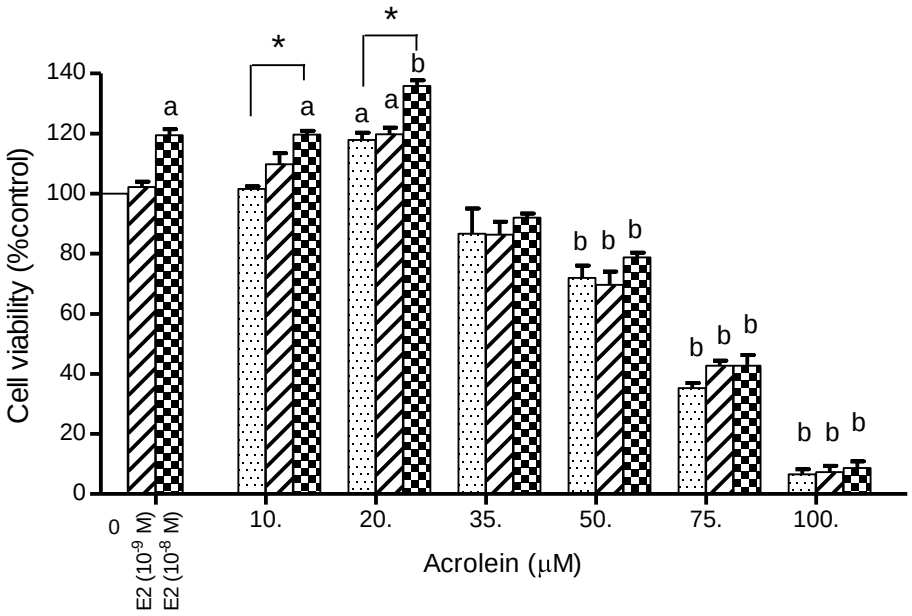
12-z cells were pre-treated with or without E2 at 10^{-9} M or 10^{-8} M for 24 h followed by addition or without addition of menadione at concentrations shown for 48 h. Cell viability was measured using the MTT assay. Values represent mean ± SEM (n=6) and are expressed as percentage of control (untreated) cells.
[a] $p<0.05$, [b] $p<0.01$, [c] $p<0.001$ vs. Control.
* $p<0.05$ menadione alone vs. menadione with 10^{-8} M E2

4.3.2.3 Effects of acrolein and E2 on cell growth and viability

Figure 4.5 reveals a significant increase in cell proliferation in the presence of 20 µM acrolein for 48 h, which reconfirmed 20 µM acrolein induced 12-z cell proliferation. The percentage of cell viability was 120% of control. However, 50 µM – 100 µM acrolein inhibited cell growth significantly.

E2 at 10^{-8} M significantly induced cell proliferation with the percentage of cell viability was 119% of control. *Figure 4.5* also demonstrates when cells were pre-treated with E2 at 10^{-8} M, a significant increased in cell proliferation was observed with addition of acrolein at 10 µM or 20 µM where the percentage of cell viability was 120% and 136% of control respectively. The viability of cells that were treated with 20 µM acrolein alone was significantly different when compared with cells that had been pre-treated with E2 at 10^{-8} M. Cell growth inhibition was observed when cells were exposed to high concentrations of acrolein at 50 µM – 100 µM despite the presence of E2.

Figure 4.5: Effects of acrolein and E2 on cell growth



12-z cells were pre-treated with or without E2 at 10^{-9} M or 10^{-8} M for 24 h followed by addition or without addition of acrolein at concentrations shown for 48 h. Cell viability was measured using the MTT assay. Values represent mean ± SEM (n=4) and are expressed as percentage of control (untreated) cells.

[a] $p<0.05$, [b] $p<0.001$ vs. Control.

* $p<0.05$ acrolein alone vs. acrolein with 10^{-8} M E2

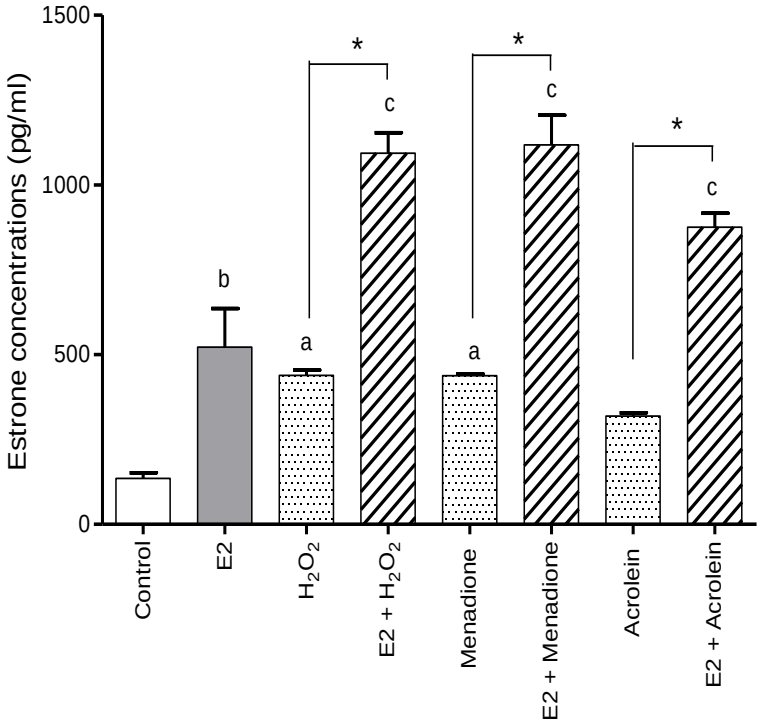
4.3.3 Effects of oxidants or acrolein and E2 on the expression or activity of estrogen biosynthesis and metabolising enzymes

This section is mainly to evaluate whether E2 production *in vitro* may be altered by oxidants, acrolein, E2 or combination of E2 and oxidants or acrolein. Moreover, it is essential to determine whether the *'Positive Feedback Loop'* that has been established in endometriotic stromal cells (Zeitoun *et al.*, 1998) may be present in endometriotic epithelial cells. Cells were treated with 1 µM H_2O_2, 20 µM menadione or 20 µM acrolein for 48 h, and 10^{-8} M E2 for 24 h. Cells were also pre-treated with 10^{-8} M E2 for 24 h followed by addition of oxidants or acrolein at similar concentrations for 48 h. These concentrations were chosen as they produced significant increases in cell proliferation.

4.3.3.1 Effects of oxidants or acrolein and E2 on estrone levels

12-z cells were plated in 24-well plates at a density of 10^4 cells/ well and allowed to attach for 24 h. Subsequently, cells were treated as above. Levels of estrone concentrations were measured by Estrone EIA as described in Chapter 2 (2.11.1). Levels of estrone concentrations were an indicator for the aromatase activity as aromatase catalyses conversion of androstenedione (A) to E1 (mainly) and E2 (Delvoux *et al.*, 2009). Therefore, the higher the estrone concentrations, the higher the aromatase activity in the cells. *Figure 4.6* shows a significant increase in estrone concentrations in cells that were treated with E2 at 10^{-8} M or oxidants alone especially combination of both. However, in acrolein treated cells, a significant increase in estrone concentrations was observed when cells were pre-treated with E2 followed by addition of acrolein. Thus, the results indicate an increase in aromatase activity especially in the presence of both E2 and oxidants or acrolein.

Figure 4.6: Effects of oxidants or acrolein and E2 on the levels of estrone concentrations



Levels of estrone concentrations in cells treated with H_2O_2 (1 µM), menadione (20 µM) or acrolein (20 µM) for 48 h and in the presence of E2 at 10^{-8} M alone for 24 h. Cells were also pre-treated with 10^{-8} M E2 for 24 h followed by addition of oxidants or acrolein at above concentrations for 48 h. Values represent mean ± SEM (n=3).
[a] $p<0.05$, [b] $p<0.01$ and [c] $p<0.001$ vs. Control
* $p<0.001$ oxidants or acrolein alone vs. oxidants or acrolein with E2

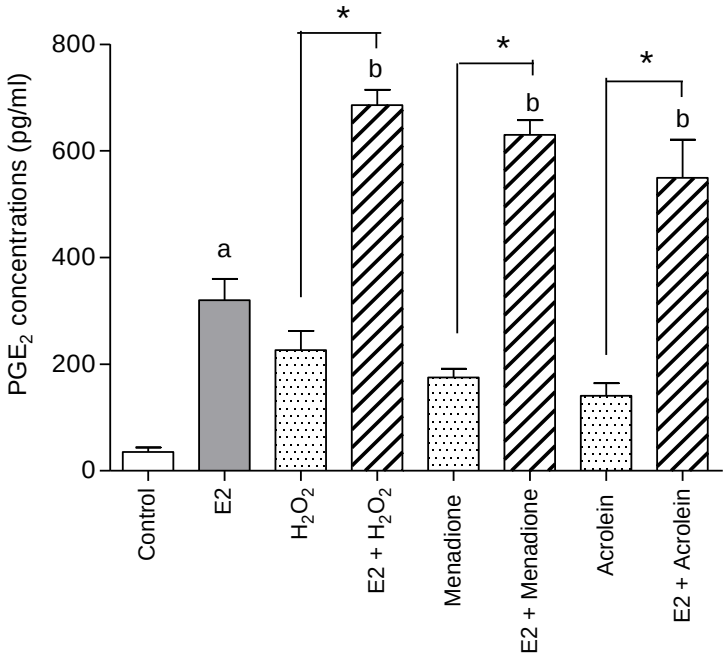
4.3.3.2 Effects of oxidants or acrolein and E2 on Prostaglandin E_2 levels

In order to determine whether the induction of aromatase was linked to a concurrent induction of PGE_2, levels of PGE_2 was measured by PGE_2 EIA as described in Chapter 2 (2.11.2). *Figure 4.7* shows a significant increase in PGE_2 levels in cells that were treated with E2 10^{-8} M alone and those cells that were pre-treated with E2 followed by addition of H_2O_2, menadione or acrolein. There is also significant difference in PGE_2 concentrations between cells that were treated with oxidants or acrolein alone, and cells that were pre-treated with E2 followed by addition of oxidants or acrolein. The results indicate PGE_2 production is stimulated by the presence of both E2 and oxidants or acrolein.

4.3.3.3 Effects of oxidants or acrolein and E2 on cyclooxygenase-2 expression

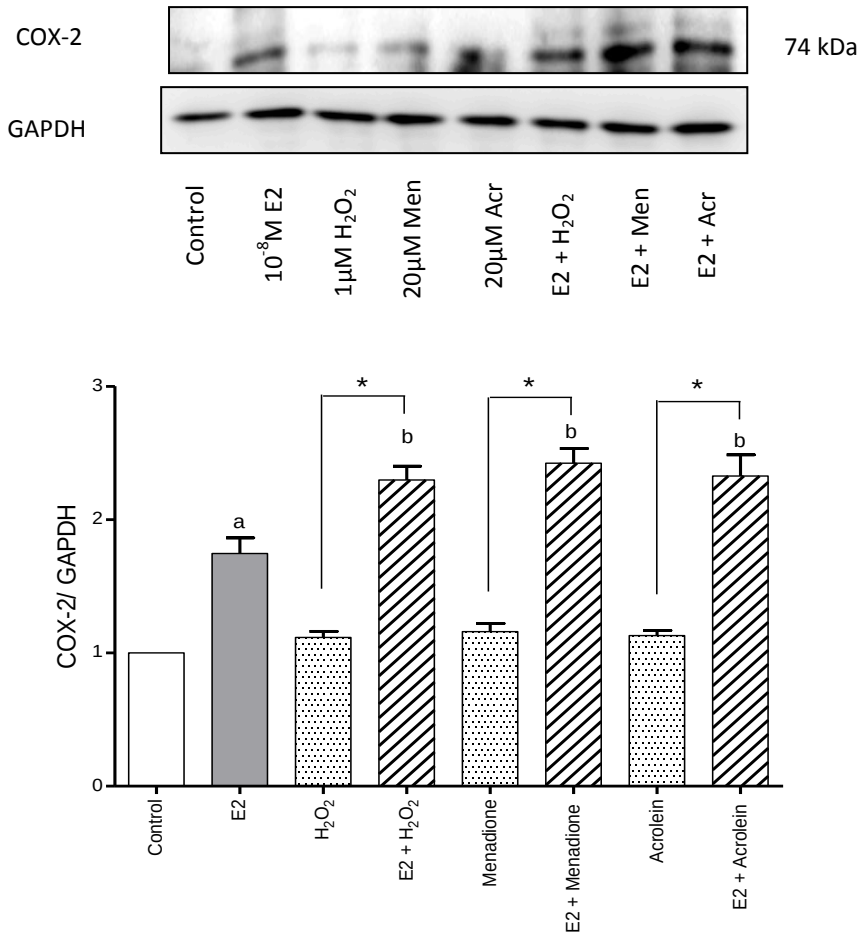
In order to assess the expression of COX-2, cells were treated as decribed earlier and COX-2 expression was determined by Western blotting using COX-2 antibodies. The results show a significant increase in COX-2 expression in cells that were treated with E2 and a further increase in the presence of E2 with H_2O_2, menadione or acrolein, as shown in *Figure 4.8*. This indicates E2 induces COX-2 expression in 12-z cells. Oxidants or acrolein alone do not induce COX-2 expression unless cells were pre-treated with E2.

Figure 4.7: Effects of oxidants or acrolein and E2 on the levels of Prostaglandin E_2



Levels of PGE_2 concentrations in cells treated with H_2O_2 (1 µM), menadione (20 µM) or acrolein (20 µM) for 48 h and in the presence of E2 at 10^{-8} M alone for 24 h. Cells were also pre-treated with 10^{-8} M E2 for 24 h followed by addition of oxidants or acrolein at above concentrations for 48 h. Values represent mean ± SEM (n=3).
[a] $p<0.01$, [b] $p<0.001$ vs. Control
* $p<0.001$ oxidants or acrolein alone vs. oxidants or acrolein with E2

Figure 4.8: Effects of oxidants or acrolein and E2 on cyclooxygenase-2 expression



Expression of COX-2 normalised with GAPDH in 12-z cells treated with H_2O_2 (1 µM), menadione (20 µM) or acrolein (20 µM) for 48 h and in the presence of E2 at 10^{-8} M alone for 24 h. Cells were also pre-treated with 10^{-8} M E2 for 24 h followed by addition of oxidants or acrolein at above concentrations for 48 h. Levels of COX-2 were analysed by Western blotting using COX-2 antibodies and GAPDH as a loading control. The protein bands were quantified by image J and expressed relative to GAPDH. Values represent mean ± SEM (n=3)

[a] $p<0.05$, [b] $p<0.001$ vs. Control.

* $p<0.001$ oxidants or acrolein alone vs. oxidants or acrolein with E2

4.3.3.4 Effects of oxidants or acrolein and E2 on 17β-HSD type 1 and 17β-HSD type 2 expressions

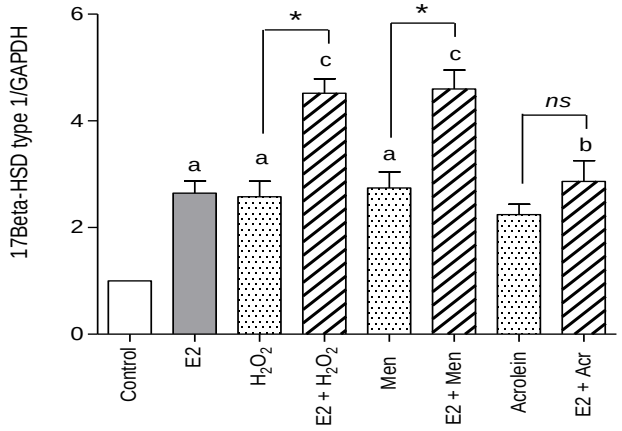
Two types of 17β-HSDs were studied here as they have opposite functions in E2 biosynthesis; 17β-HSD type 1 catalyses the activation of E1 to E2 whereas 17β-HSD type 2 inactivates E2 to E1. In this study, mRNA levels of both 17β-HSD isoforms were determined. 17β-HSD type 1 and 17β-HSD type 2 mRNA were quantified by q-RT-PCR using 17β-HSD type 1 and 17β-HSD type 2 oligonucleotides.

Figure 4.9(a) shows a significant increase in 17β-HSD type 1 mRNA levels in cells that were treated with E2, H_2O_2 and menadione alone with the fold increases were 2.64, 2.57 and 2.87 respectively when compared to control. The increase in 17β-HSD type 1 mRNA levels were higher when cells were pre-treated with E2 at 10^{-8} M followed by addition of H_2O_2 or menadione with the fold increases were 4.52 and 4.60 respectively. Although acrolein alone did not induce 17β-HSD type 1 mRNA expression, the fold significantly increases to 2.87 as compared to control when cells were pre-treated with E2. The levels of these enzymes were also significantly difference between oxidants alone and E2 with oxidants.

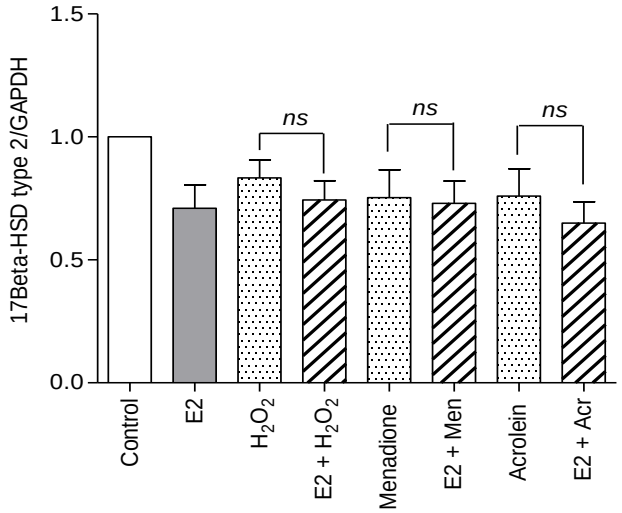
Conversely, there was no significant difference in 17β-HSD type 2 mRNA levels in all treated cells as compared to control as shown in *Figure 4.9(b)*. Therefore, 17β-HSD type 1, but not 17β-HSD type 2 plays an important role in E2 biosynthesis.

Figure 4.9: Effects of oxidants or acrolein and E2 on 17β-HSD type 1 and 17β-HSD type 2 expressions

a)



b)



Expression of a) 17β-HSD type 1 and b) 17β-HSD type 2 mRNA normalised with GAPDH in 12-z cells treated with H_2O_2 (1 µM), menadione (20 µM) or acrolein (20 µM) for 48 h and in the presence of E2 at 10^{-8} M alone for 24 h. Cells were also pre-treated with 10^{-8} M E2 for 24 h followed by addition of oxidants or acrolein at above concentrations for 48 h. Levels of mRNA were quantified by normalisation to the GAPDH as an internal control. Values represent mean ± SEM (n=3).

[a] $p<0.05$, [b] $p<0.01$ and [c] $p<0.001$ vs. Control

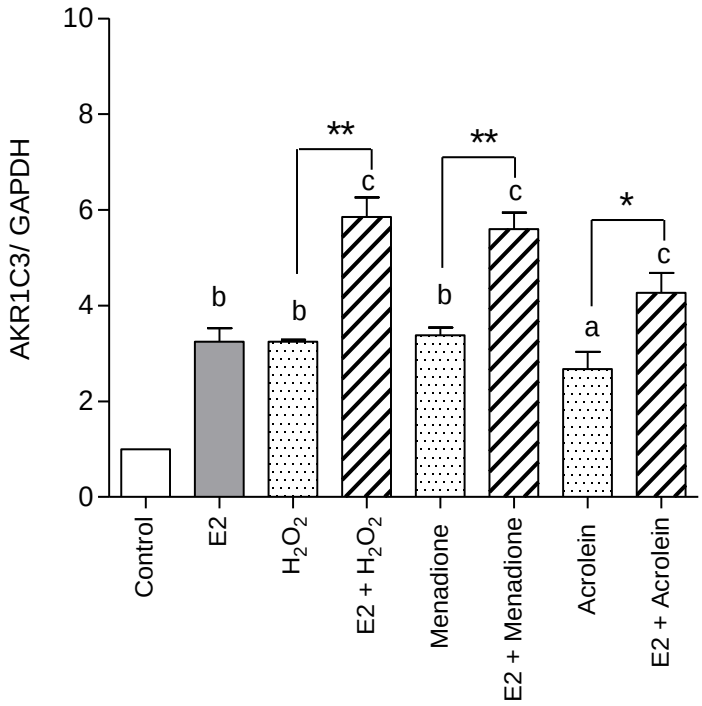
* $p<0.01$ oxidants alone vs. oxidants with E2

ns: not significant

4.3.3.5 Effects of oxidants or acrolein and E2 on AKR1C3 expression

Although the function of this enzyme is mainly to catalyse active progesterone to the less active 20α-hydroxyprogesterone (20α-OHP), it is considered to be examined in this study as it is also involved in E2 biosynthesis. Similarly, cells were treated with E2 for 24 h or H_2O_2, menadione or acrolein alone for 48 h, and cells were also pre-treated with E2 at 10^{-8} M for 24 h, followed by addition of H_2O_2, menadione or acrolein for another 48 h. AKR1C3 mRNA was quantified by q-RT-PCR using AKR1C3 oligonucleotides. *Figure 4.10* shows a significant increase in the AKR1C3 mRNA levels in all treated cells with the fold increases were 3.24, 3.23, 3.37 and 2.68 in cells that were treated with E2, H_2O_2, menadione or acrolein respectively when compared to control. In addition, the levels of AKR1C3 mRNA are much higher when cells were pre-treated with E2 before the addition of H_2O_2, menadione or acrolein with the fold increases were 5.85, 5.6 and 4.27 respectively.

Figure 4.10: Effects of oxidants or acrolein and E2 on AKR1C3 expression



Expression of AKR1C3 mRNA normalised with GAPDH in 12-z cells treated with H_2O_2 (1 µM), menadione (20 µM) or acrolein (20 µM) for 48 h and in the presence of E2 at 10^{-8} M alone for 24 h. Cells were also pre-treated with 10^{-8} M E2 for 24 h followed by addition of oxidants or acrolein at above concentrations for 48 h. Levels of mRNA were quantified by normalisation to the GAPDH as an internal control. Values represent mean ± SEM (n=3).

[a] $p<0.05$, [b] $p<0.01$ and [c] $p<0.001$ vs. Control

* $p<0.05$, ** $p<0.01$ oxidants or acrolein alone vs. oxidants or acrolein with E2

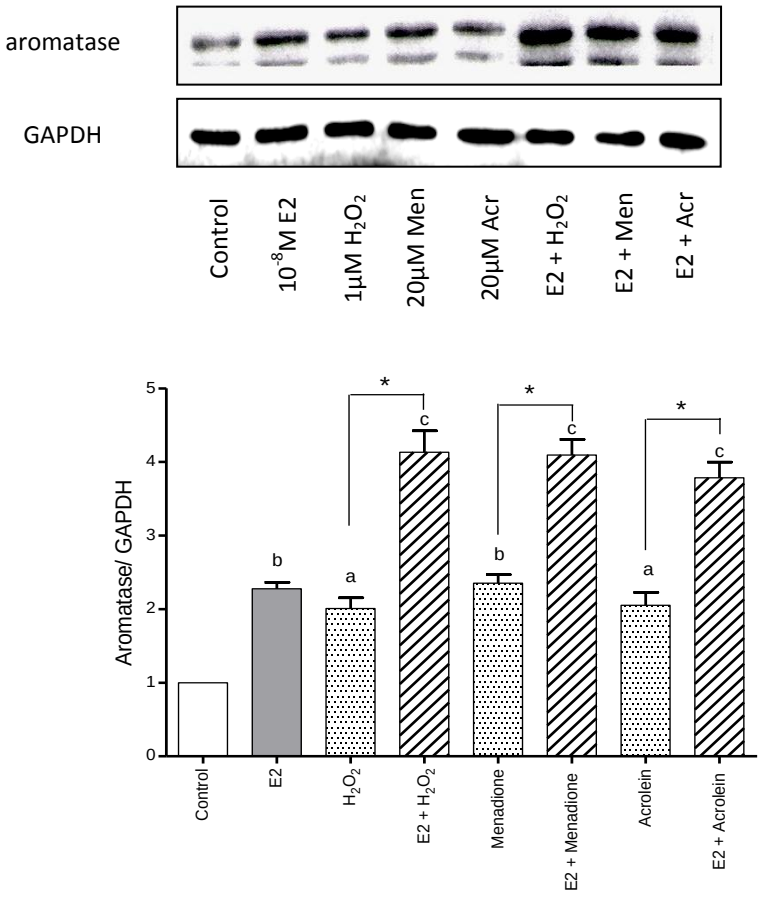
4.3.4 Effects of oxidants or acrolein and E2 on aromatase expression

The expression of aromatase, a key enzyme for E2 synthesis, was assessed whether it responds to oxidants, acrolein or E2. Cells were treated as described earlier and aromatase expression was determined by Western blotting using aromatase antibodies. The results show a significant increase in aromatase expression in all treated cells especially in the presence of both E2 and H_2O_2, menadione or acrolein as compared to control, as shown in *Figure 4.11*. This indicates that E2, oxidants and acrolein and especially combination of both E2 and oxidants or acrolein significantly induced aromatase expression in 12-z cells.

4.3.5 Aromatase expression in human samples

To determine whether an increase levels in aromatase expression in endometriosis as one of the possible causes of increasing in cell proliferation, aromatase mRNA levels were determined in samples from patients with ovarian endometriosis and *Uterine myomatosus* as controls. *Figure 4.12* shows a significant increase in aromatase expression in ovarian endometriosis as compared to controls. Although this study proposed aromatase to have an important role in endometriosis, the sample size was too small to infer the levels in the population. A limited number of patients with endometriosis is one of the possible causes of the study limitation. In future, a large number of samples which requires a longer period of study for sample collection, needs to be considered in order to make a valid inference about the aromatase levels in patients with and without endometriosis.

Figure 4.11: Effects of oxidants or acrolein and E2 on aromatase expression

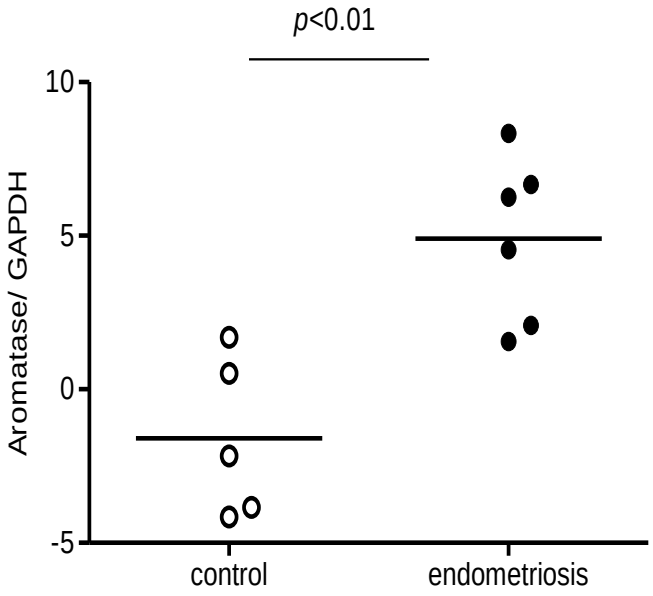


Expression of aromatase normalised with GAPDH in 12-z cells treated with H_2O_2 (1 µM), menadione (20 µM) or acrolein (20 µM) for 48 h and in the presence of E2 at 10^{-8} M alone for 24 h. Cells were also pre-treated with 10^{-8} M E2 for 24 h followed by addition of oxidants or acrolein at above concentrations for 48 h. Levels of aromatase were analysed by Western blotting using aromatase antibodies and GAPDH as a loading control. The protein bands were quantified by image J and expressed relative to GAPDH. Values represent mean ± SEM (n=3).

[a] $p<0.05$, [b] $p<0.01$ and [c] $p<0.001$ vs. Control.

* $p<0.001$ oxidants or acrolein alone vs. oxidants or acrolein with E2

Figure 4.12: Expression of aromatase mRNA in endometriotic cells and controls.



Expression of aromatase mRNA in endometriotic cells and controls. The fold changes of mRNA levels were quantified by normalisation to the GAPDH as an internal control, which detected by q-RT-PCR. Values represent mean (n=5; control and n=6; ovarian endometriosis)

4.3.6 Effects of oxidants or acrolein and E2 on the localisation of estrogen receptors

Study to determine which ERs being the main regulator or mediator of E2 action is important to understand the mechanism of cell proliferation. The localisation of receptors following a stimulus may determine which receptor/s is activating. In order to evaluate the role of E2 receptors in mediating the observed E2 effects, ER-α and ER-β were determined in the nuclear and cytosolic fractions in 12-z cells that were treated with E2 for 24 h or H_2O_2, menadione or acrolein alone for 48 h, and cells were also pre-treated with E2 at 10^{-8} M for 24 h, followed by addition of H_2O_2, menadione or acrolein for another 48 h.

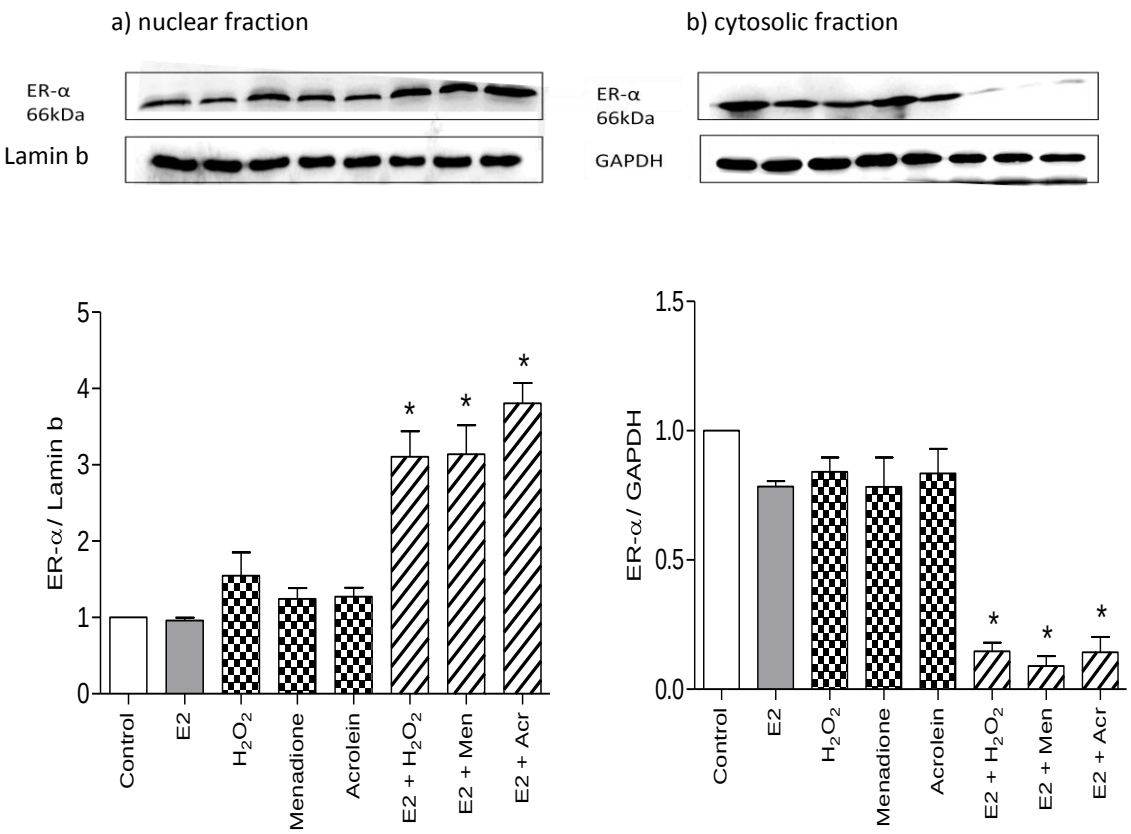
4.3.6.1 Estrogen Receptor-α

A significant increase in nuclear ER-α levels was observed in cells that were pre-treated with E2 at 10^{-8} M followed by addition of H_2O_2, menadione or acrolein with the fold increases were 3.11, 3.14 and 3.80 when compared to control, as shown in *Figure 4.13(a)*. These increases were found to correlate with the corresponding decrease in cytosolic ER-α as seen in *Figure 4.13(b)*. Thus, both E2 and oxidants or acrolein activate the translocation of ER-α from the cytosol to the nucleus.

4.3.6.2 Estrogen Receptor-β

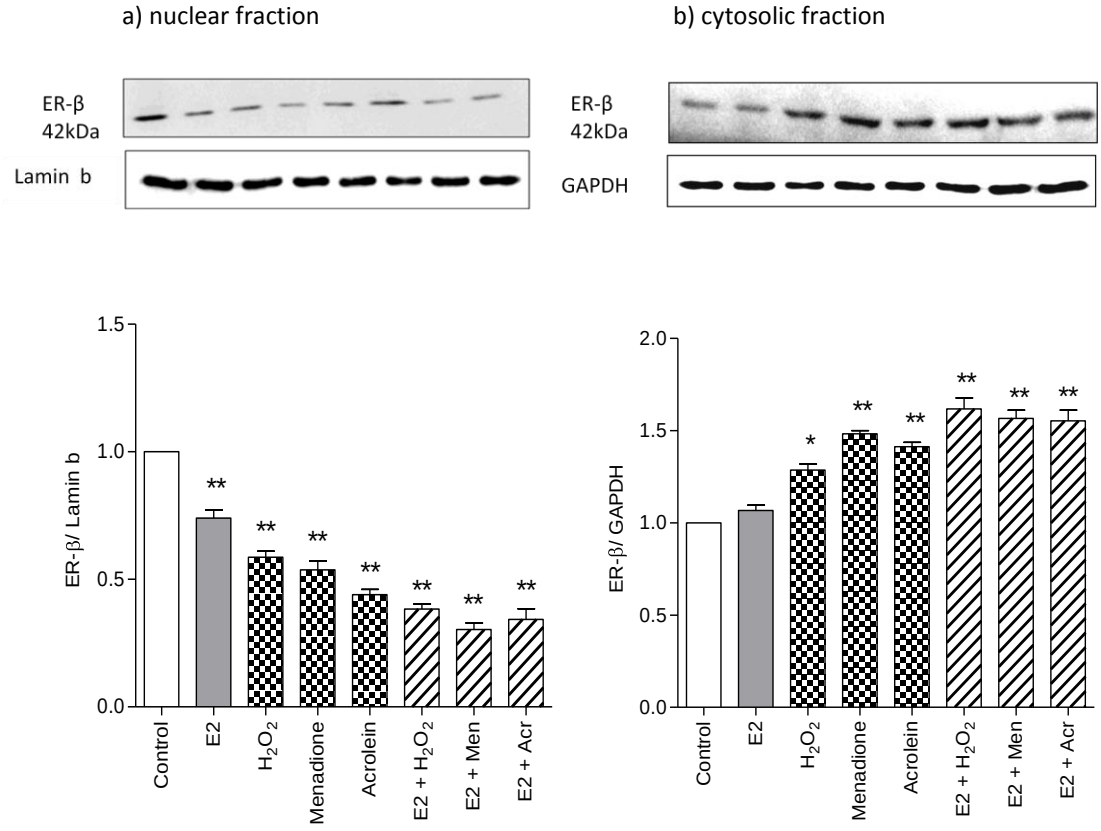
Comparing to control, a significant decrease in nuclear ER-β levels was observed in all treated cells, as shown in *Figure 4.14(a)*. These decreases were found to correlate with the corresponding increase in cytosolic ER-β as seen in Figure *4.14(b)*. These data indicate that ER-β did not induce by E2, oxidants or acrolein, thus they are accumulated in cytosol.

Figure 4.13: Effects of oxidants or acrolein and E2 on ER-α localisation



Localisation of ER-α in a) nuclear fraction and b) cytosolic fraction normalised with Lamin b and GAPDH respectively in 12-z cells with H_2O_2 (1 µM), menadione (20 µM) or acrolein (20 µM) for 48 h and in the presence of E2 at 10^{-8} M alone for 24 h. Cells were also pre-treated with 10^{-8} M E2 for 24 h followed by addition of oxidants or acrolein at above concentrations for 48 h. Levels of ER-α enzyme were analysed by Western blotting using ER-α antibodies and Lamin b or GAPDH as a loading control. The protein bands were quantified by image J. Values represent mean ± SEM (n=3). * p<0.001 vs. Control.

Figure 4.14: Effects of oxidants or acrolein and E2 on ER-β localisation



Localisation of ER-β in a) nuclear fraction and b) cytosolic fraction normalised with Lamin b and GAPDH respectively in 12-z cells with H_2O_2 (1 µM), menadione (20 µM) or acrolein (20 µM) for 48 h and in the presence of E2 at 10^{-8} M alone for 24 h. Cells were also pre-treated with 10^{-8} M E2 for 24 h followed by addition of oxidants or acrolein at above concentrations for 48 h. Levels of ER-β enzyme were analysed by Western blotting using ER-β antibodies and Lamin b or GAPDH as a loading control. The protein bands were quantified by image J. Values represent mean ± SEM (n=3). $^{*}p<0.05$, $^{**}p<0.001$ vs. Control.

4.3.7 Effects of oxidants or acrolein and E2 on estrogen receptors mRNA level

4.3.7.1 Estrogen Receptor-α

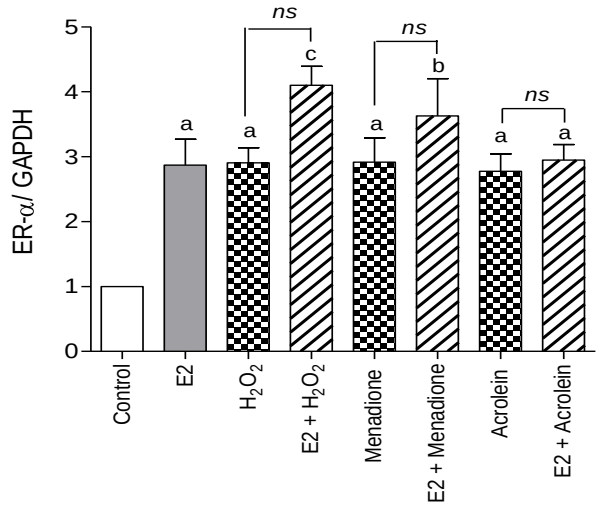
Figure 4.15(a) shows a significant increase in ER-α mRNA levels in all treated cells as compared to control. The fold increases were 2.87, 2.90, 2.91 and 2.77 in cells that were treated with E2, H_2O_2, menadione and acrolein respectively. The fold increases were more obvious when cells were pre-treated with E2 followed by addition of H_2O_2 and menadione with the fold increases were 4.10 and 3.63 respectively when compared to control.

4.3.7.2 Estrogen Receptor-β

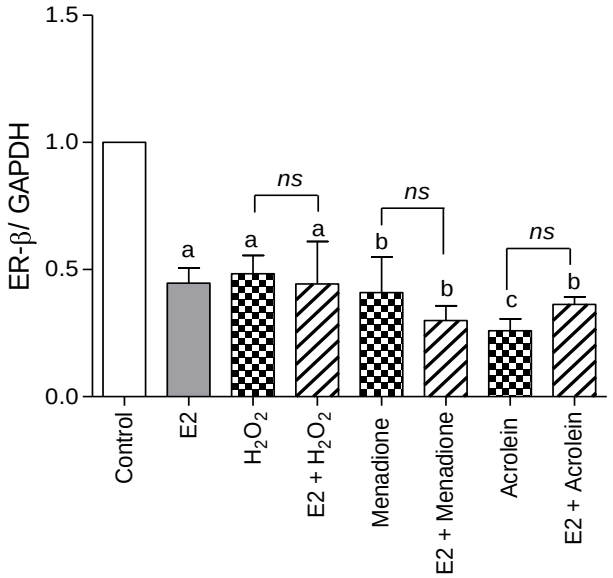
In contrast, *Figure 4.15(b)* shows a significant decrease in ER-β mRNA levels in all treated cells as compared to control.

Figure 4.15: Effects of oxidants or acrolein and E2 on ER-α and ER-β mRNA levels

a)



b)



Expression of a) ER-α and b) ER-β mRNA normalised with GAPDH in 12-z cells treated with H_2O_2 (1 µM), menadione (20 µM) or acrolein (20 µM) for 48 h and in the presence of E2 at 10^{-8} M alone for 24 h. Cells were also pre-treated with 10^{-8} M E2 for 24 h followed by addition of oxidants or acrolein at above concentrations for 48 h. Levels of mRNA were quantified by normalisation to the GAPDH as an internal control. Values represent mean ± SEM (n=3).
[a] $p<0.05$, [b] $p<0.01$, [c] $p<0.001$ vs. Control.
ns: not significant

4.4 Discussion

4.4.1 Combination of E2 and oxidants or acrolein induced endometriotic cell proliferation

E2 is the main estrogenic hormone, which plays an essential roles in a wide variety of biological processes including reproduction, differentiation, cell proliferation, apoptosis, inflammation, metabolism, homeostasis and brain function (Marino *et al.*, 2006). E2 at a concentration of 10^{-8} M significantly induced cell proliferation as compared to control. The proliferative effects of H_2O_2, menadione and acrolein at low concentrations were reconfirmed in this chapter. Similar to the previous results (Chapter 3), at high levels of these substances, the inhibitory effects of the cells were clearly demonstrated. E2, especially at a concentration of 10^{-8} M seems to play an important role by increasing the effects of oxidants and acrolein induced cell proliferation. The effects between E2 and oxidants or acrolein are more additive than synergistic. An additive effect is the sum of the two compounds individually whereas synergistic effect will give a greater response than the sum of the parts. Interestingly, 10^{-8} M E2 was able to increase the percentages of viable cells significantly when treated with lower concentrations of H_2O_2, menadione and acrolein than above, i.e. 0.5 µM H_2O_2, 15 µM menadione and 10 µM acrolein. The present results suggest E2, especially with the combination of oxidants or acrolein enhanced endometriotic cell proliferation. Thus, it has to be determined what other factors that involved in the mechanism of endometriotic cell proliferation.

4.4.2 Roles of estrogen biosynthesis and metabolising enzymes in endometriosis

The biosynthesis and bioavailability, or the net production of estrogen depends on the balance between the production of active estrogens and their inactivation, which is determined by the activity of key enzymes. Various types of estrogen biosynthesis and metabolising enzymes have been studied by many researchers and

in this study, the expression of five enzymes were studied; aromatase, COX-2, 17β-HSD type 1, 17β-HSD type 2, and AKR1C3. Compared to the previously published data that mainly used normal endometrium as controls, this study however used untreated endometriotic cells. The focus of this study is to determine the changes in gene expression in endometriotic cells when exposed to various types of environment or assuming the subsequent effects of endometriotic cells in patients that have different peritoneal fluid environment.

4.4.2.1 Roles of aromatase in endometriosis

The expression of aromatase was determined in 12-z cells by Western blotting and it appears that E2, oxidants and acrolein induce its expression. The combination of E2 with oxidants or acrolein aggravates the expression. This condition mimics the physiological situation in peritoneal fluid where endometriotic stromal cells have been reported to produce E2 locally (Attar & Bulun, 2006a) and high levels of ROS have been found in the peritoneal fluid of women with endometriosis (Wang *et al.*, 1997). In this study, the pivotal role of aromatase is further supported by the results of q-RT-PCR showed that aromatase mRNA expression in endometriotic cells from samples of ovarian endometriosis was significantly higher as compared to controls, which consist of samples from patients with *Uterine myomatosus.* An increase in aromatase expression is suggested to be responsible for increase production of E2 thus maintaning endometriotic cell growth and proliferation.

4.4.2.2 Aromatase activity in endometriosis

The increased expression of aromatase in treated 12-z cells correlates well with aromatase activity. In this study, Estrone EIA has been used to determined the aromatase activity, i.e by measuring the estrone concentrations as aromatase catalyses conversion of androstenedione to mainly estrone, similar to previous

studies that determined aromatase activity on 45 chemicals, which have been reported as endocrine disruptors (Satoh *et al.*, 2008). These authors reported that the sensitivity of estrone EIA was 10 times higher compared to the radio-isotope method. The present study revealed high levels of estrone concentrations in treated 12-z cells especially in the presence of both E2 and oxidants or acrolein. There were also significant differences in estrone concentrations between oxidants or acrolein alone and E2 plus oxidants or acrolein.

4.4.2.3 Roles of Prostaglandin E_2 in endometriosis

Besides estrogen metabolising enzymes, the present study determined PGE_2 concentrations in 12-z cells as this factor has been suggested as the most potent inducer of aromatase activity in endometriotic cells (Noble *et al.*, 1997). Previous studies that used 12-z cells and human immortalized endometriotic stromal cells have found abundantly expressed of PGE_2 in both types of the cells as compared with human immortalized endometrial epithelial cells and human immortalized endometrial stromal cells, which were measured by EIA (Banu *et al.*, 2008). Using the same technique (EIA), others have determined the expression of PGE_2 was significantly increased in endometrial stromal cells that were cultured with the peritoneal fluid from women with endometriosis compared to peritoneal fluid from normal women (Liu *et al.*, 2011).

It is rather interesting that the results were similar to COX-2 where PGE_2 concentrations were significantly higher in cells that were treated with 10^{-8} M E2 alone and the concentrations were further increased in the presence of both 10^{-8} M E2 and oxidants or acrolein. This may be explained by the facts that COX-2 is known to up-regulate PGE_2 formation although it has been shown in endometriotic stromal cells (Zeitoun *et al.*, 1999). With regards to an increase in aromatase activity especially in cells that were treated with both E2 and oxidants or acrolein, high

levels of PGE_2 concentrations in these groups of cell may explain the increase aromatase activity as PGE_2 induces aromatase activity.

4.4.2.4 Roles of 17β-HSD type 1 and type 2 in endometriosis

In humans, aromatase produces mainly estrone, which needs to be converted to E2, an active form of estrogen (Bulun *et al.*, 2002b). Although several reductive enzymes including 17β-HSD type 1, 7 and 12 have been reported previously to catalyse the activation of E1 to E2 (Smuc & Rizner, 2009), only 17β-HSD type 1 has been measured in this study. The data demonstrated that E2, H_2O_2 and menadione alone significantly induced 17β-HSD type 1 mRNA expression and this was further increased in the presence of both E2 and oxidants. However, acrolein alone did not increase the mRNA levels of this enzyme unless cells were pre-treated with 10^{-8} M E2. Therefore, the combination of both E2 and oxidants or acrolein will increase E2 production, which is mediated through 17β-HSD type 1. Previous studies have also found a significant increase in 17β-HSD type 1 mRNA expression in samples from ovarian endometriosis as compared to samples from patients with *Uterine myomatosus* as control (Smuc *et al.*, 2009).

Different types of oxidative enzymes that catalyse the inactivation of E2 to E1 have been reported such as 17β-HSD type 2, 4, 8, 10 and 14 (Smuc & Rizner, 2009). In this chapter, only 17β-HSD type 2 has been investigated and the results revealed no significant difference in any of the treated cell as compared to control similar to previous studies (Smuc *et al.*, 2009). Similar to 17β-HSD type 1, samples from women with *Uterine myomatosus* were used as control. Conversely, 17β-HSD type 2 was found to be absent in endometriotic cells (Zeitoun *et al.*, 1998). Previous studies that used the same cells as in this study and endometriotic stromal cells reported that the expression of 17β-HSD type 2 was barely detectable (Banu *et al.*, 2008). The reason for the discrepancy in 17β-HSD type 2 levels remains unclear, particularly when similar methodologies were used. The low levels of this enzyme

in the present study may explain the reason for the increase in E2 in endometriotic cells as E2 is not converted to estrone, which would lead to an increase in cell proliferation.

4.4.2.5 Roles of cyclooxygenase-2 in endometriosis

The expression of COX-2 was determined in the present study using Western blotting. COX-2 expression was found to be induced significantly in cells that were treated with 10^{-8} M E2 and the expression was much higher in cells that were pre-treated with 10^{-8} M E2 followed by addition of oxidants or acrolein. However, in the presence of oxidants or acrolein only, there was no significant difference in the expression of COX-2 as compared to control. A number of investigators have shown that COX-2 expression can be induced in endometriotic stromal cells by various factors such as E2 (Zeitoun *et al.*, 1999), IL-6, IL-11 and tumour necrosis factor (TNF)-α (Bulun *et al.*, 1999), IL-1β (Tamura *et al.*, 2002b) and VEGF (Tamura *et al.*, 2002a). Most recently, levels of COX-2 mRNA were found to be significantly higher in endometrial stromal cells that were cultured with the peritoneal fluid from women with endometriosis compared to peritoneal fluid from normal women (Liu *et al.*, 2011). Although many studies seem to propose this enzyme has a pivotal role in endometriosis, others have found an equal level of this enzyme in both eutopic endometrium and ectopic endometriotic tissue of women with endometriosis (Wu *et al.*, 2005). The results of COX-2 expression in this study indicate E2 as the main factor that induce COX-2 expression.

4.4.2.6 Roles of AKR1C3 in endometriosis

The expression of one of the 20α-HSDs, AKR1C3 was investigated in this study using q-RT-PCR. Oxidants, acrolein and E2 alone significantly increase AKR1C3 mRNA expression and the expression was further increased when cells were pre-treated

with E2 10^{-8} M. Therefore, E2 especially with oxidants or acrolein induced the expression of AKR1C3 genes. AKR1C3 possess 17β-HSD activity that catalyses the conversion of estrone to E2, which will further increase the levels of E2 (Penning *et al.*, 2000). Recently, studies have shown a significant up-regulation of AKR1C3 mRNA in ovarian endometriotic cells as compared to samples from women with *Uterine myomatosus* tissue as control (Smuc *et al.*, 2009).

The results that have been demonstrated so far, suggest that the mechanism of cell proliferation in 12-z cells is due to continuous E2 production, which is achieved by interrelated function of enzymes or factors that have been mentioned. It suggests that the additive effects of E2 and oxidants or acrolein lead to significant induction of certain genes although acrolein has been shown to exert less effect. This is the first *in vitro* study that has looked at the direct effects of oxidants or acrolein on the expression of aromatase, PGE_2, COX-2, 17β-HSDs type 1 and 2, and AKR1C3 in endometriotic epithelial or stromal cells.

4.4.3 Roles of estrogen receptors in endometriosis

The abundance and distribution of ERs and progesterone receptors (PRs) will determine whether E2 or progesterone will give a particular effect. In order for E2 to exert its effects, the presence of ERs, ER-α and/ or ER-β is needed. In this study, both ER-α and ER-β were examined to evaluate whether they have a role in mediating E2 effects in 12-z cells. In contrast to other genes, the expression of these receptors was determined in nuclear and cytosolic fractions. The expression and localisation of both receptors were determined by Western blotting and q-RT-PCR. Estrogen action is mediated by interaction with its receptors in the cytosol in the presence of stimuli (Walters, 1985). Ligand binding promotes the receptors which then move in the cell nucleus where they interact with estrogen response elements, ERE in the promoter regions of their target genes to initiate the biological response (Verderame & Limatola, 2010). The details of the mechanism of estrogen

action have been described in Chapter 1. Therefore, free receptor is located in the cytosol whereas steroid-bound receptor is associated with the nuclear fraction.

The results show a significant increase in nuclear ER-α level in cells that were treated with both E2 at 10^{-8} M and H_2O_2 or menadione or acrolein. In addition, these increases were found to correlate with the corresponding decrease in cytosolic ER-α. These data indicate that in the presence of both E2 and oxidants or acrolein, there is translocation of ER-α from the cytosol to the nucleus. On the other hand, the ER-β expression was significantly low in the nuclear fractions, but high in the cytosolic fractions in all treated cells as compared to control.

The q-RT-PCR of ER-α revealed that E2, oxidants and acrolein upregulated its expression significantly, which was further increased when cells were pre-treated with E2. However, ER-β expression was significantly low in all treated cells as compared to control, which suggest the compounds used in this study did not induce its expression. Although transcription levels do not always correspond to the protein levels (Rizner, 2009), the q-RT-PCR results is consistent with the Western blotting, which indicate ER-α as the main receptor that mediate the E2 action. Since the development of endometriosis requires cell proliferation, it is conceivable that E2 through ER-α may regulate cell proliferation in this disease. In contrast to the findings of this study, ER-α has been found to be down-regulated whereas ER-β to be up-regulated in endometriotic cells (Bukulmez *et al.*, 2008; Smuc *et al.*, 2009). In that study, q-RT-PCR was used to determine the expression of both receptors, which compare endometriotic cells and normal endometrium. In 12-z cells, it has been shown that ER-α was abundantly expressed whereas ER-β was weakly expressed, that has been demonstrated on 1% agarose gel (Banu *et al.*, 2008).

In summary, this chapter has implicated the additive effects of E2 and oxidants or acrolein in the mechanism of endometriotic cell proliferation. The up-regulation of

most of the genes is mainly due to combination of E2 and oxidants (mainly) or acrolein. ER-α is suggested to have a pivotal role in endometriotic cell proliferation. The results of the present study for the first time demonstrate another *'positive feedback loop'* for continuous E2 production that involves E2, oxidants and acrolein, particularly in endometriotic epithelial cells as shown in *Figure 4.16*.

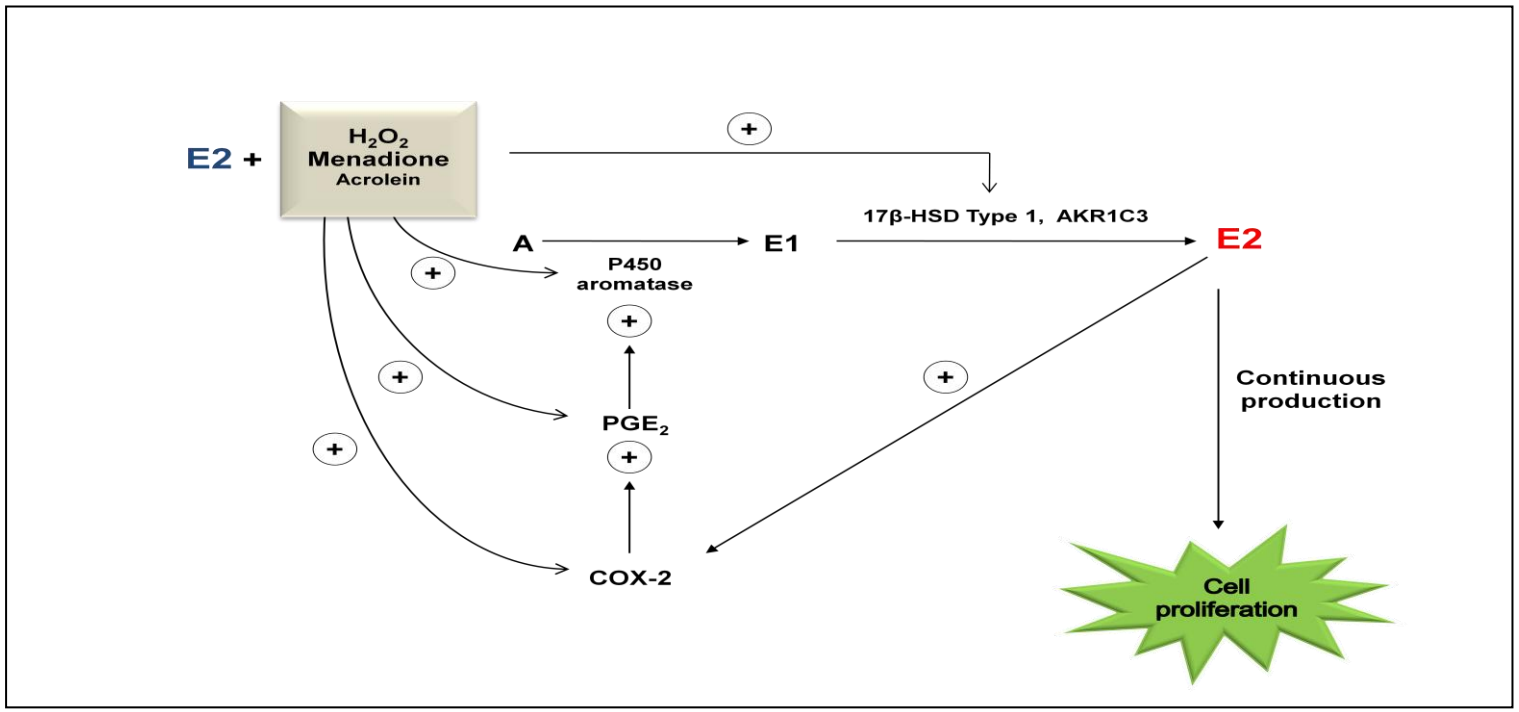


Figure 4.16: Diagram showing proposed mechanism of *'Positive Feedback Loop'* for continuous E2 production in 12-z cells. E2 and oxidants or acrolein (less effects), stimulate COX-2 expression, which further increased PGE_2 production. PGE_2 production activates aromatase activity that produce high amount of estrone. 17β-HSD type 1 and AKR1C3 convert E1 to E2. This process will be continuous and results in increase of E2 synthesis in 12-z cells.

E2: endogenous E2

E2: exogenous E2

A:androstenedione, E1: Estrone. E2: Estradiol, COX-2: cyclooxygenase-2, PGE_2: prostaglandin E_2.

CHAPTER FIVE:

ROLES OF SIGNALING PATHWAYS IN ESTRADIOL, OXIDANTS AND ACROLEIN INDUCED ENDOMETRIOTIC CELL PROLIFERATION

5.1 Introduction

The results of previous chapters (Chapter 3 & Chapter 4) demonstrated that E2, oxidants, acrolein and especially the combination of E2 and oxidants or acrolein induced cell proliferation in 12-z endometriotic cells. In this chapter, the possible involvement of signaling pathway/s in the mechanism of endometriotic cell proliferation is investigated. Identifying which signals are activated in the mechanism of endometriotic cell proliferation and growth, are of considerable interest. In endometriosis, there are two signaling pathways that have been studied most, mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/ serine/threonine protein kinase (Akt) pathways.

Previous studies have also used 12-z cells to determine roles of signaling pathways in the mechanism of cell proliferation in endometriosis. Grund *et al*., have demonstrated MAPK/ERK1/2 and p38 MAPK signaling pathways are involved in the pathogenesis of endometriosis by using 12-z cells (Grund *et al.*, 2008). This showed that TNF-α induced phosphorylation of both MAPK/ERK1/2 and p38 MAPK signaling that was blocked by their inhibitors, PD98059 and SB203580 respectively. The expression of inflammatory biomarkers was also determined including IL-6, IL-8 and granulocyte macrophage-colony-stimulating factor (GM-CSF) in these cells that were treated with TNF-α. IL-6 is known as a chemoattractant for monocytes whereas IL-8 activates angiogenesis and neutrophil migration and differentiation, and GM-CSF stimulates granulocyte and monocyte differentiation (Grund *et al.*, 2008). The results revealed that PD98059 and SB203580 blocked TNF-α-induced the secretion of IL-6, IL-8 and GM-CSF. The interruption of TNF-α-induced MAPK/ERK1/2 and p38 MAPK signaling pathways in endometriotic cells decreased the secretion of biomarkers for inflammation that resulted in delaying the disease progression. Conversely, these authors have reported that PI3K signaling pathways may not be involved in the pathogenesis of this disease as PI3K inhibitor

(Wortmannin) did not inhibit TNF-α-induced the secretion of IL-6, IL-8 and GM-CSF in 12-z cells, which have been shown by MAPK/ERK1/2 and p38 MAPK pathways. Therefore, the roles of MAPK pathways mainly involves the changes in the levels of inflammatory biomarkers, which suggests that the mechanism of endometriotic cell proliferation is mainly due to inflammatory process that occur in endometriotic cells. Previous studies on the involvement of MAPK and PI3K/Akt signaling pathways in the mechanism of endometriotic cell proliferation have been described in Chapter 1 (section 1.5).

5.1.1 Chapter Aims

Despite several lines of evidence for the involvement of different signaling pathways in the mechanism of endometriotic cell proliferation, the exact factors that activate the pathway/s are still inconclusive. Whether the role of E2 in the mechanism of endometriotic cell proliferation also involves the non ERE-dependent pathway is needs to be determined. With evidence implicating the involvement of signaling pathways in the mechanism of cell proliferation, it is essential to identify factors that activate the pathways that are involved in cell proliferation in endometriosis. The identification of signaling pathways and also the factors activations may lead to valuable therapeutic approaches for limiting disease progression.

Therefore, the objectives of the present study are:

To determine the activity of MAPK/ERK1/2 and PI3K/AKT signaling pathways and their possible roles in regulating endometriotic cell proliferation caused by E2, oxidants and acrolein.

5.2 Materials and methods

Inhibitors:

Two inhibitors (purchased from Sigma, UK) were used; i. PD98059 (Chemical name: 2-(2-Amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one) and ii. LY294002 (Chemical name: 2-(4-morpholinyl)-8-phenylchromone) to determine the involvement of MAPK/ERK1/2 and PI3K/Akt signaling pathways respectively.

i. PD98059

The solution was prepared as below:

1 mg of PD98059 (MW=267.3) was dissolved in 187 µl DMSO to prepare the stock solution (20 mM). An amount of 20 µl of 20 mM PD98059 was made up to 20 ml with culture media to prepare 20 µM PD98059 working solution.

ii. LY294002

The solution was prepared as below:

5 mg of LY294002 (MW=343.8) was dissolved in 294 µl DMSO to prepare the stock solution 5 mM. An amount of 40 µl of 5 mM LY294002 was made up to 20 ml with culture media 10 µM LY294002 working solution.

All other materials and methods for this chapter are described in chapter 2.

5.3 Results

5.3.1 MAPK/ERK1/2 activation

To evaluate MAPK/ERK1/2 as the intracellular signaling pathway of endometriotic cell proliferation, cells were treated with oxidants, acrolein or E2 at different duration of culture. The regulation of this pathway by those compounds was examined. Controls were untreated cells that had been cultured for 24 h.

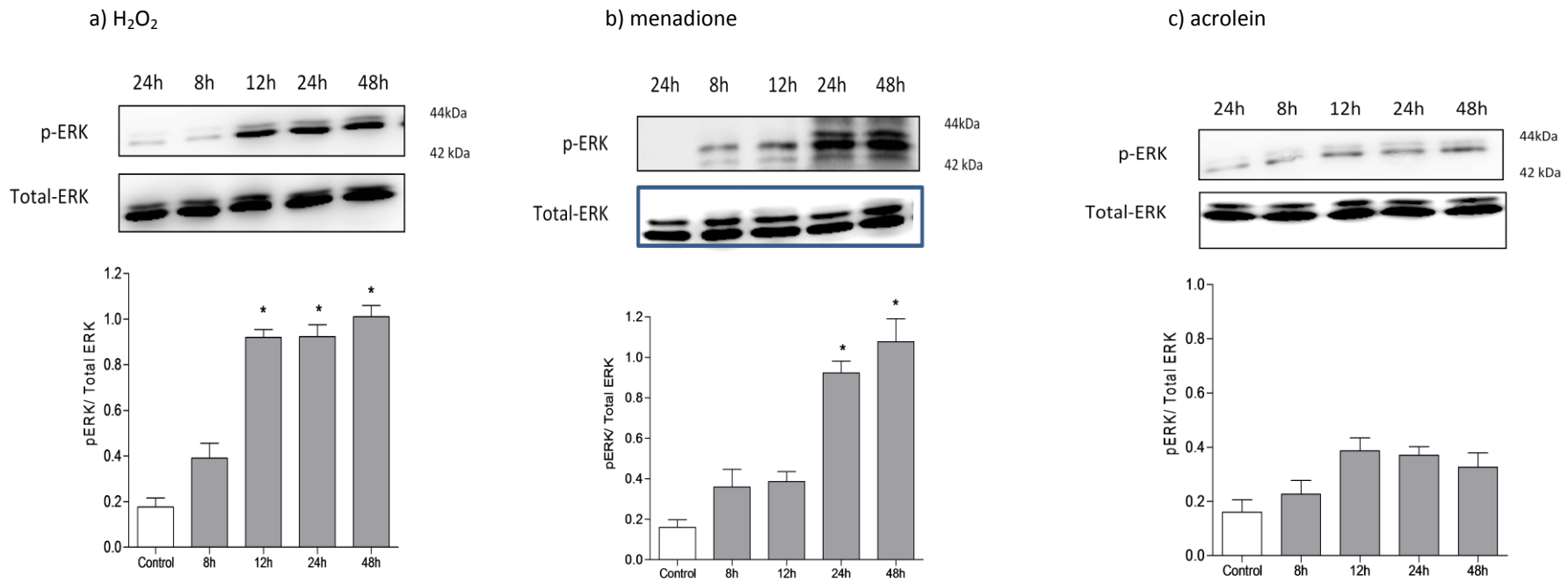
5.3.1.1 MAPK/ERK1/2 activation in response to oxidants and acrolein

Cells were treated with 1 µM H_2O_2, 20 µM menadione or 20 µM acrolein for 8 h, 12 h, 24 h and 48 h. These concentrations gave a significant increase in cell proliferation from previously. The phosphorylation of ERK1/2 was detected by Western blotting. The ratio of phospho(p)-ERK1/2/ total-ERK was significantly higher in cells that were treated with 1 µM H_2O_2 within 12 h (*Figure 5.1a*) and 20 µM menadione within 24 h (*Figure 5.1b*) as compared to control. However, as shown in *Figure 5.1c*, the acrolein-induced ERK1/2 phosphorylation was marginal even until 48 h of culture. Total ERK1/2 remained constant in 12-z cells, treated and untreated.

5.3.1.2 MAPK/ERK1/2 activation in response to E2

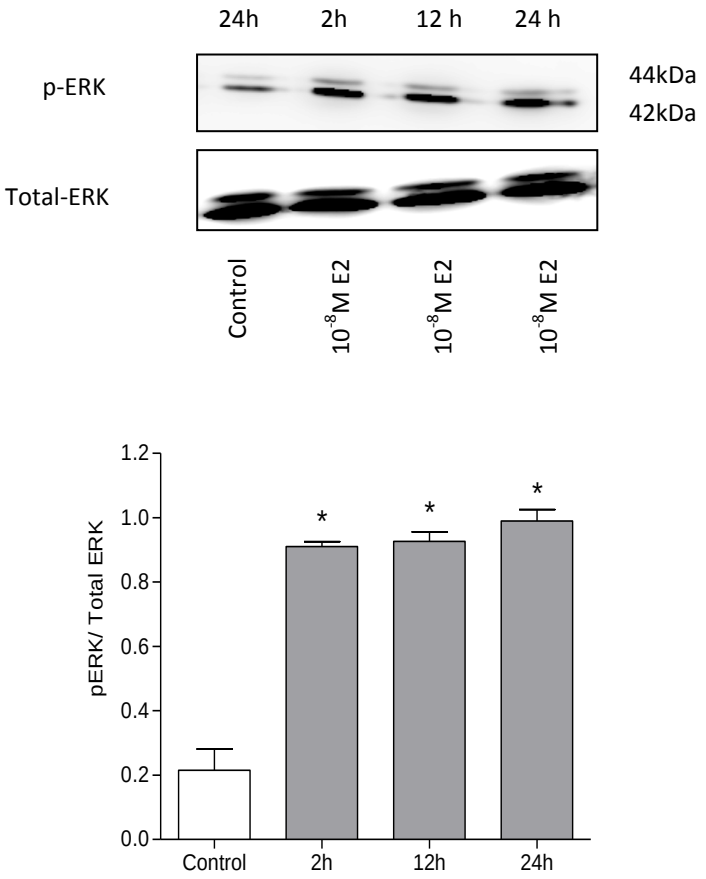
Cells were treated with 10^{-8} M E2 for 2 h, 12 h and 24 h. *Figure 5.2* revealed a significant upregulation of p-ERK1/2/ total ERK was observed as early as 2 h of culture and sustained for at least 24 h of culture.

Figure 5.1: Effects of oxidants and acrolein on the phosphorylation of MAPK/ERK1/2



Cells were treated with a) H_2O_2 (1 µM), b) menadione (20 µM) or c) acrolein (20 µM) for 8 h, 12 h, 24 h and 48 h. The expression of p-ERK1/2 and total ERK was determined by Western blotting using Phospho-p44/42 MAPK (ERK1/2) antibodies and p44/42 MAPK (ERK1/2) as a loading control. The protein bands were quantified by image J. Values represent mean ± SEM (n=3).
*p<0.001 vs. Control.

Figure 5.2: Effects of E2 on the phosphorylation of MAPK/ERK1/2



Cells were treated with E2 at 10^{-8} M for 2 h, 12 h and 24 h. The expression of p-ERK1/2 and total ERK was determined by Western blotting using Phospho-p44/42 MAPK (ERK1/2) antibodies and p44/42 MAPK (ERK1/2) as a loading control. The protein bands were quantified by image J. Values represent mean ± SEM (n=3).
*p<0.001 vs. Control.

5.3.1.3 Effects of PD98059 on MAPK/ERK1/2 activation in response to oxidants, acrolein and E2

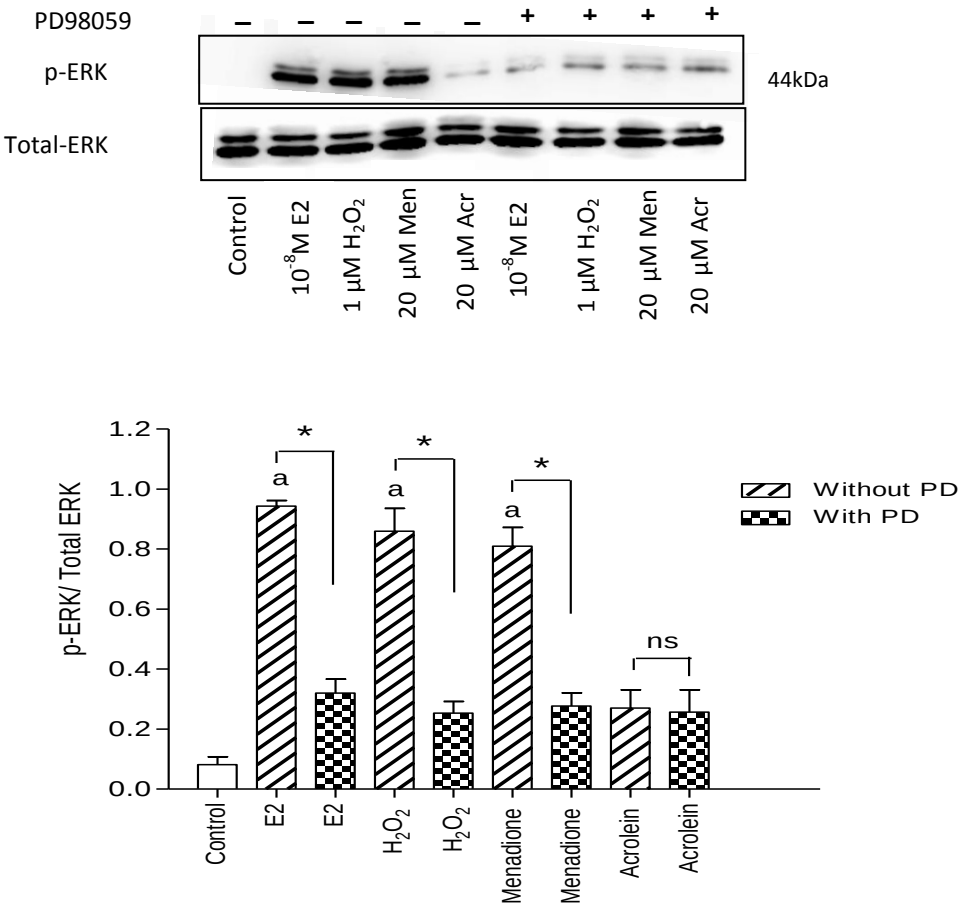
Cells were pre-treated with or without 20 µM PD98059 for 1 h followed by addition of 1 µM H_2O_2, 20 µM menadione or 20 µM acrolein for 48 h, and 10^{-8} M E2 for 24 h. *Figure 5.3* shows a significant increase in p-ERK/total ERK ratio in cells that were treated with H_2O_2, menadione and E2, which was inhibited when cells were pre-treated with 20 µM of PD98059. PD98059 is a specific inhibitor of MEK1, which blocks phosphorylation and activation of MAPK proteins, ERK1 and ERK2 (Long *et al.*, 2001).

5.3.1.4 Effects of PD98059 on MAPK/ERK1/2 activation in response to combination of E2 and oxidants or acrolein

Cells were pre-treated with 10^{-8} M E2 for 24 h followed by addition of 1 µM H_2O_2, 20 µM menadione or 20 µM acrolein for 48 h. To determine the effects of inhibitor, cells were exposed to inhibitor for 1 h before or after addition of 10^{-8} M E2.

Figure 5.4a demonstrates a significant increase in p-ERK/total ERK ratio in cells treated with E2 alone and in combination with H_2O_2, menadione or acrolein as compared to control. However, the p-ERK/total ERK ratio was not significantly different between E2 and its combination with oxidants or acrolein. In the presence of PD98059 after E2 exposure, the p-ERK1/2 was partially inhibited as only E2 induced the phosphorylation. However, there was a complete inhibition of p-ERK1/2, when PD98059 was added before cells were exposed to any of the compounds, as shown in *Figure 5.4b and 5.4c*.

Figure 5.3: Effects of PD98059 on the E2, oxidants or acrolein induced phosphorylation of MAPK/ERK1/2



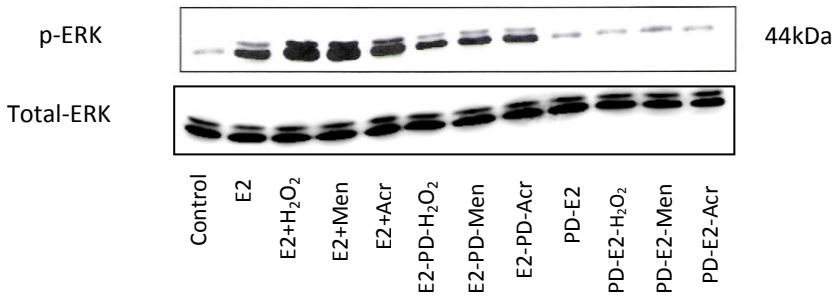
Cells were pre-treated with and without 20 µM PD98059 for 1 h followed by addition of 10^{-8} M E2 for 24 h, 1 µM H_2O_2, 20 µM menadione or 20 µM acrolein for 48 h. The expression of p-ERK1/2 and total ERK was determined by Western blotting using Phospho-p44/42 MAPK (ERK1/2) antibodies and p44/42 MAPK (ERK1/2) as a loading control. The protein bands were quantified by image J. Values represent mean ± SEM (n=3).

[a] $p<0.001$ vs. Control

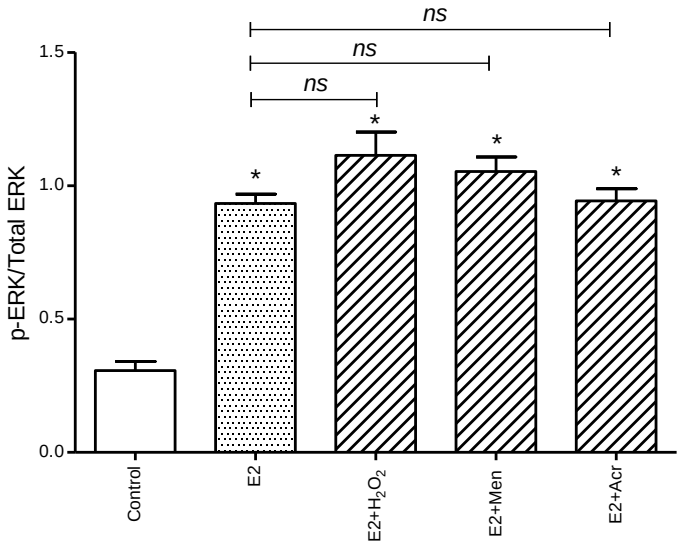
* $p<0.001$ E2 or oxidants alone vs. oxidants or E2 with PD

ns: not significant

Figure 5.4: Effects of PD98059 on E2 alone or combination of E2 and oxidants or acrolein induced phosphorylation of MAPK/ERK1/2



a)

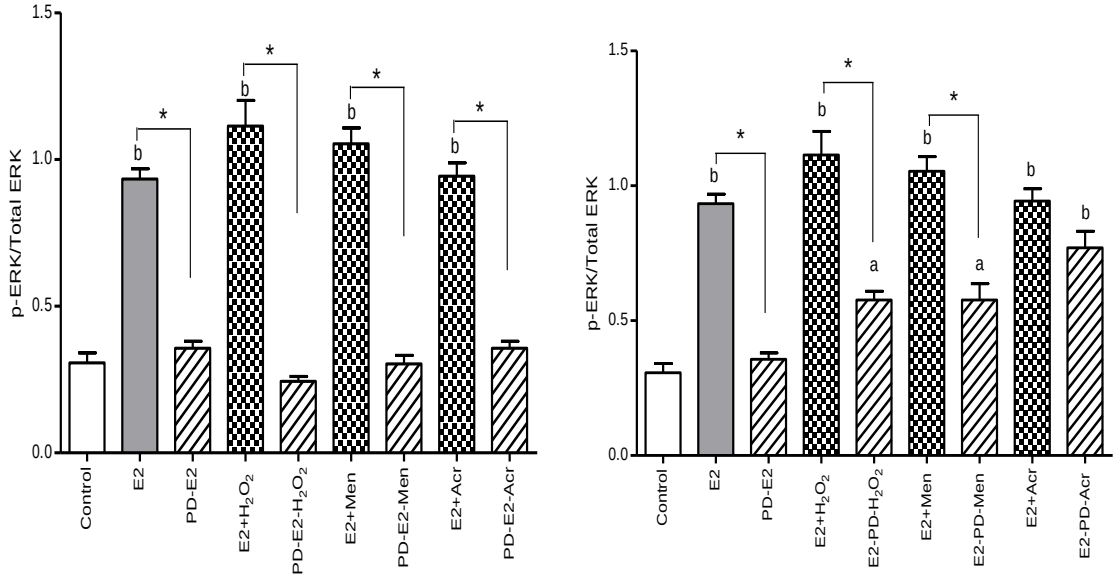


Cells were treated with 10^{-8} M E2 alone for 24 h or combination with 1 µM H_2O_2, 20 µM menadione or 20 µM acrolein for 48 h. The expression of p-ERK1/2 and total ERK was determined by Western blotting using Phospho-p44/42 MAPK (ERK1/2) antibodies and p44/42 MAPK (ERK1/2) as a loading control. The protein bands were quantified by image J. Values represent mean ± SEM (n=3).

* $p<0.05$ vs. Control

ns: not significant

b) c)



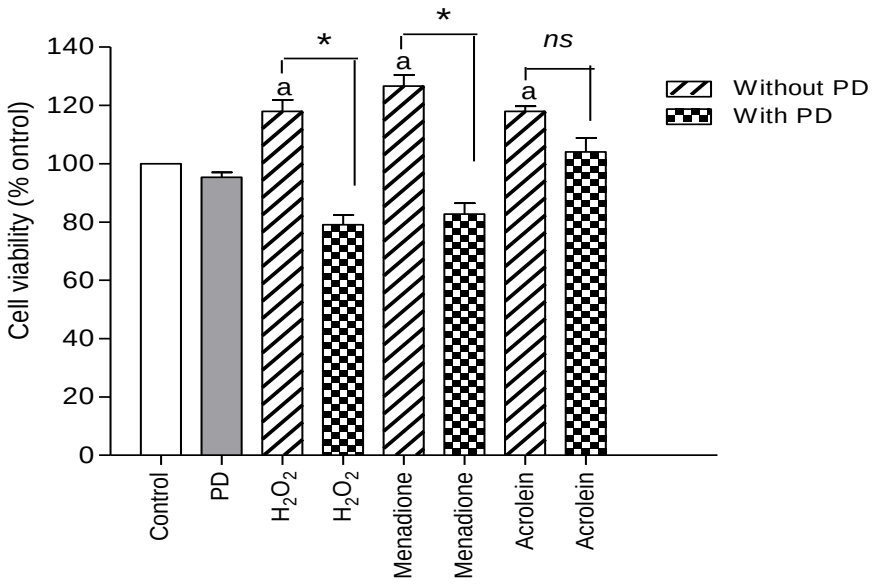
Cells were treated with 10^{-8} M E2 alone for 24 h or combination with 1 µM H_2O_2, 20 µM menadione or 20 µM acrolein for 48 h. Cells were also exposed to 20 µM PD98059 for 1 h before (b) or after (c) addition of 10^{-8} M E2. The expression of p-ERK1/2 and total ERK was determined by Western blotting using Phospho-p44/42 MAPK (ERK1/2) antibodies and p44/42 MAPK (ERK1/2) as a loading control. The protein bands were quantified by image J. Values represent mean ± SEM (n=3).
[a] $p<0.05$, [b] $p<0.001$ vs. Control
* $p<0.001$ E2, oxidants or acrolein alone vs. E2, oxidants or acrolein with PD

Addition of inhibitor before E2:	PD-E2-oxidants or acrolein
Addition of inhibitor after E2:	E2-PD-oxidants or acrolein

5.3.1.5 Effects of PD98059 on oxidants and acrolein induced cell proliferation

To further determine whether MAPK/ERK1/2 is involved in the mechanism of 12-z endometriotic cell proliferation, cell viability MTT assay was done. Cells were pre-treated with or without 20 µM PD98059 for 1 h followed by addition of 1 µM H_2O_2, 20 µM menadione and 20 µM acrolein for 48 h. As shown in *Figure 5.5*, a significant upregulation of cell viability was observed with the percentages were 115%, 126%, 118% for H_2O_2, menadione and acrolein respectively as compared to control. It is interesting to note that the observed cell proliferation in H_2O_2 and menadione treated cells was inhibited by PD98059 with a significant reduction of 36% and 43% respectively, but not acrolein.

Figure 5.5: Effects of PD98059 on oxidants and acrolein induced cell proliferation



Cells were pre-treated with or without 20 µM PD98059 for 1 h followed by addition of 1 µM H_2O_2, 20 µM menadione or 20 µM acrolein for 48 h at the concentrations shown. Cell viability was measured using the MTT assay. Values represent mean ± SEM (n=6) and are expressed as percentage of control (untreated) cells.

[a] $p<0.05$ vs. Control

* $p<0.05$ oxidants alone vs. oxidants with PD

ns: not significant

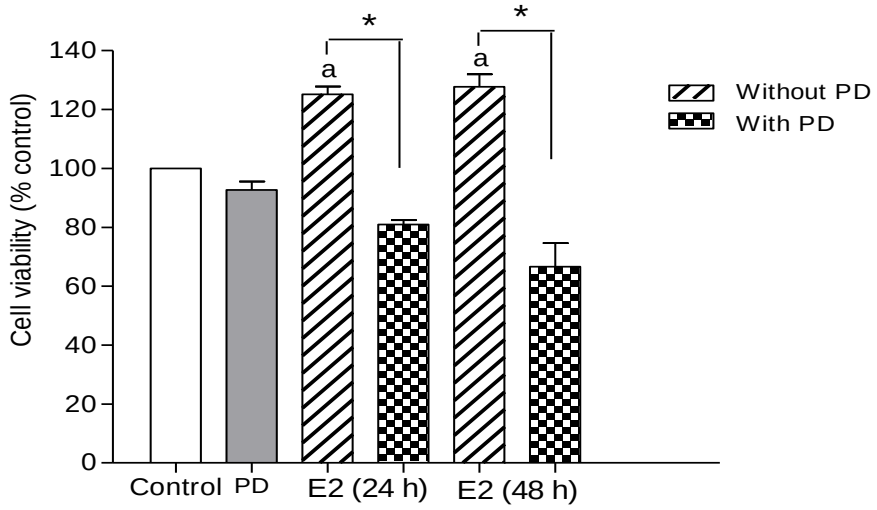
5.3.1.6 Effects of PD98059 on E2 induced cell proliferation

To further determine whether E2 mediates the regulation of MAPK/RK1/2 pathway in 12-z cells, MTT assay was done. Cells were treated with E2 at 10^{-8} M alone for 24 h and 48 h with or without pre-treatment with 20 µM PD98059. As shown in *Figure 5.6*, 10^{-8} M E2 significantly increased cell proliferation, with cell viability of 125% and 128% at 24 h and 48 h respectively as compared to control. A significant reduction of cell growth was observed when cells were pre-treated with inhibitor even cells were allow to grow for 48 h. The percentage of cell viability was 56% and 59% in cells treated with E2 for 24 h and 48 h respectively.

5.3.1.7 Effects of PD98059 on combination of E2 and oxidants or acrolein induced cell proliferation

Cells were pre-treated with 10^{-8} M E2 for 24 h followed by addition of 1 µM H_2O_2, 20 µM menadione or 20 µM acrolein for 48 h. Cells were also exposed to 20 µM PD98059 for 1 h before or after addition of 10^{-8} M E2. Combination of both E2 and oxidants or acrolein significantly increased cell proliferation with the percentages of cell viability were 130%, 123% and 120% for E2 with H_2O_2, E2 with menadione, or E2 with acrolein respectively, as shown in *Figure 5.7a, b and c*. A significant reduction of cell viability by PD98059 was observed as compared to cells treated with E2 alone or E2 with oxidants. It is proposed that PD98059 inhibits E2 and oxidants induced cell proliferation whereas the effects of this inhibitor are less apparent with acrolein.

Figure 5.6: Effects of PD98059 on E2 induced cell proliferation

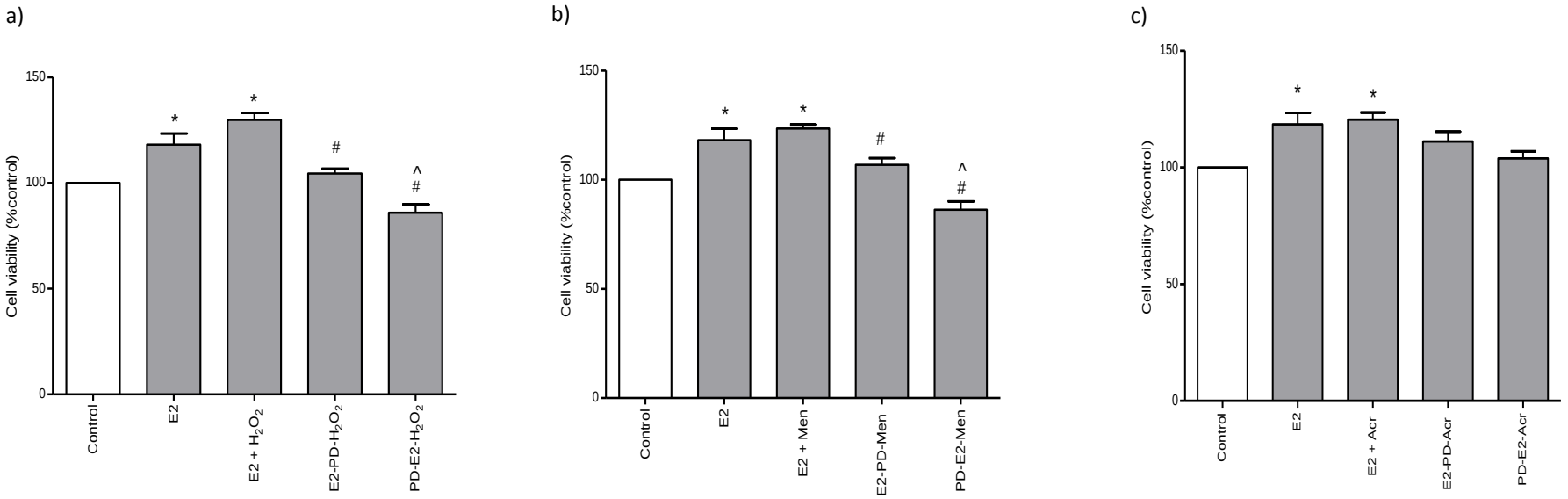


Cells were pre-treated with and without 20 µM PD98059 for 1 h followed by addition of E2 at 10^{-8} M for 24 and 48 h. Cell viability was measured using the MTT assay. Values represent mean ± SEM (n=6) and are expressed as percentage of control (untreated) cells.

[a] $p<0.05$ vs. Control

* $p<0.001$ E2 alone vs. E2 with PD

Figure 5.7: Effects of PD98059 on E2 alone and E2 with oxidants or acrolein induced cell proliferation



Cells were treated with 10^{-8} M E2 alone for 24 h or combination with a) 1 µM H_2O_2 b) 20 µM menadione or c) 20 µM acrolein for 48 h. Cells were also exposed to 20 µM PD98059 for 1 h before or after addition of 10^{-8} M E2. Cell viability was measured using the MTT assay. Values represent mean ± SEM (n=6) and are expressed as percentage of control (untreated) cells.

* $p<0.05$ vs. Control

^ $p<0.05$ vs E2 alone

# $p<0.05$ vs E2 with oxidants

Addition of inhibitor before E2:	PD-E2-oxidants or acrolein
Addition of inhibitor after E2:	E2-PD-oxidants or acrolein

5.3.2 PI3K/Akt activation

In order to determine whether PI3K/Akt signaling pathway is involved in the mechanism of endometriotic cell proliferation, cells were treated with oxidants, acrolein or E2 at different duration of culture. Controls were untreated cells that were cultured for 24 h.

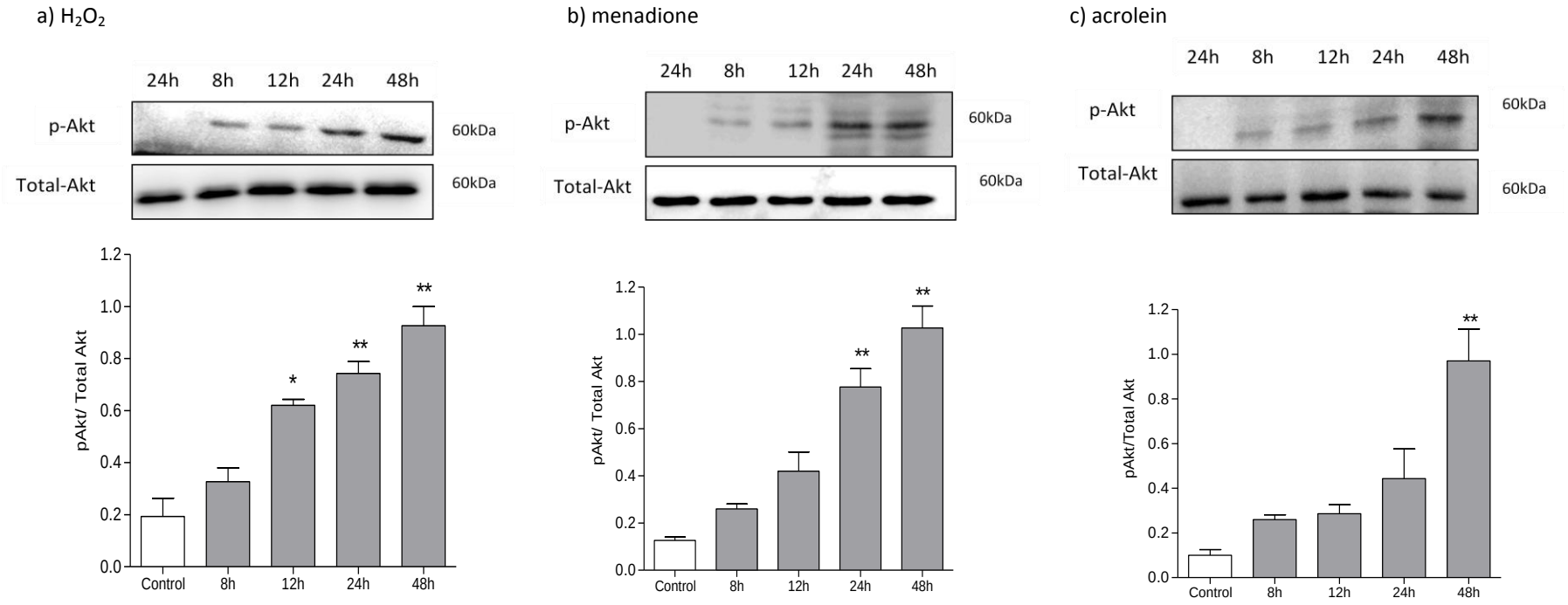
5.3.2.1 PI3K/Akt activation in response to oxidants and acrolein

Cells were treated with 1 µM H_2O_2, 20 µM menadione or 20 µM acrolein for 8 h, 12 h, 24 h and 48 h, the concentrations that gave a significant increase in cell proliferation from previously. The phosphorylation of Akt was detected by Western blotting at 8 h, 12 h, 24 h and 48 h. As shown in *Figure 5.8a, b and c*, the ratio of phospho (p)-Akt/ total-Akt was significantly higher in cells treated with 1 µM H_2O_2 within 24 h, 20 µM menadione within 12 h and 20 µM acrolein after 48 h of culture. However, total Akt remained constant in 12-z cells, treated and untreated.

5.3.2.2 PI3K/Akt activation in response to E2

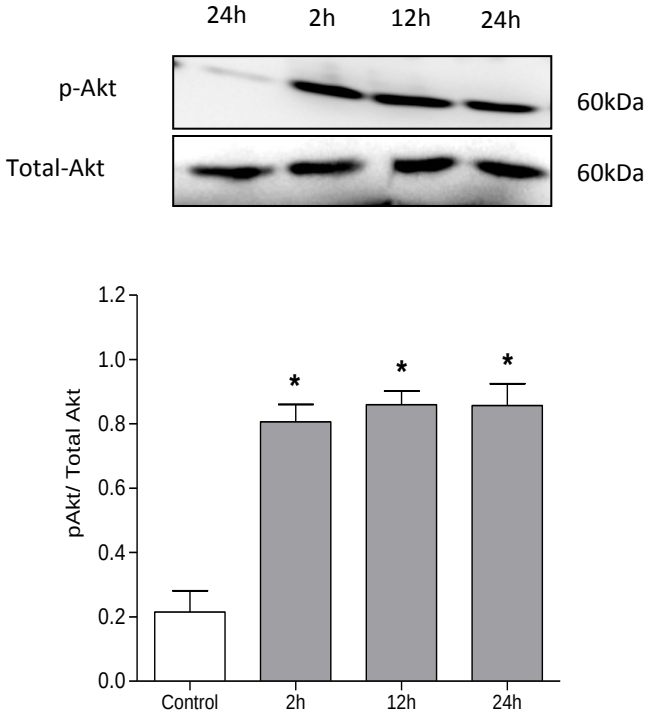
In addition, as shown in *Figure 5.9*, a significant activation of p-Akt was observed in cells that were treated with E2 at 10^{-8} M as early as 2 h of culture and sustained for at least 24 h.

Figure 5.8: Effects of oxidants and acrolein on the phosphorylation of PI3K/Akt



Cells were treated with a) H_2O_2 (1 µM), b) menadione (20 µM) or c) acrolein (20 µM) for 8 h, 12 h, 24 h and 48 h. The expression of p-Akt and total Akt was determined by Western blotting using Phospho-Akt (Ser473) antibodies and Akt as a loading control. The protein bands were quantified by image J. Values represent mean ± SEM (n=3).
*p<0.01, **p<0.001 vs. Control.

Figure 5.9: Effects of E2 on the phosphorylation of PI3K/Akt



Cells were treated with E2 at 10^{-8} M for 2 h, 12 h and 24 h. The expression of p-Akt and total Akt was determined by Western blotting using Phospho-Akt (Ser473) antibodies and Akt as a loading control. The protein bands were quantified by image J. Values represent mean ± SEM (n=3).
*p<0.001 vs. Control.

5.3.2.3 Effects of LY294002 on PI3K/Akt activation in response to E2, oxidants and acrolein

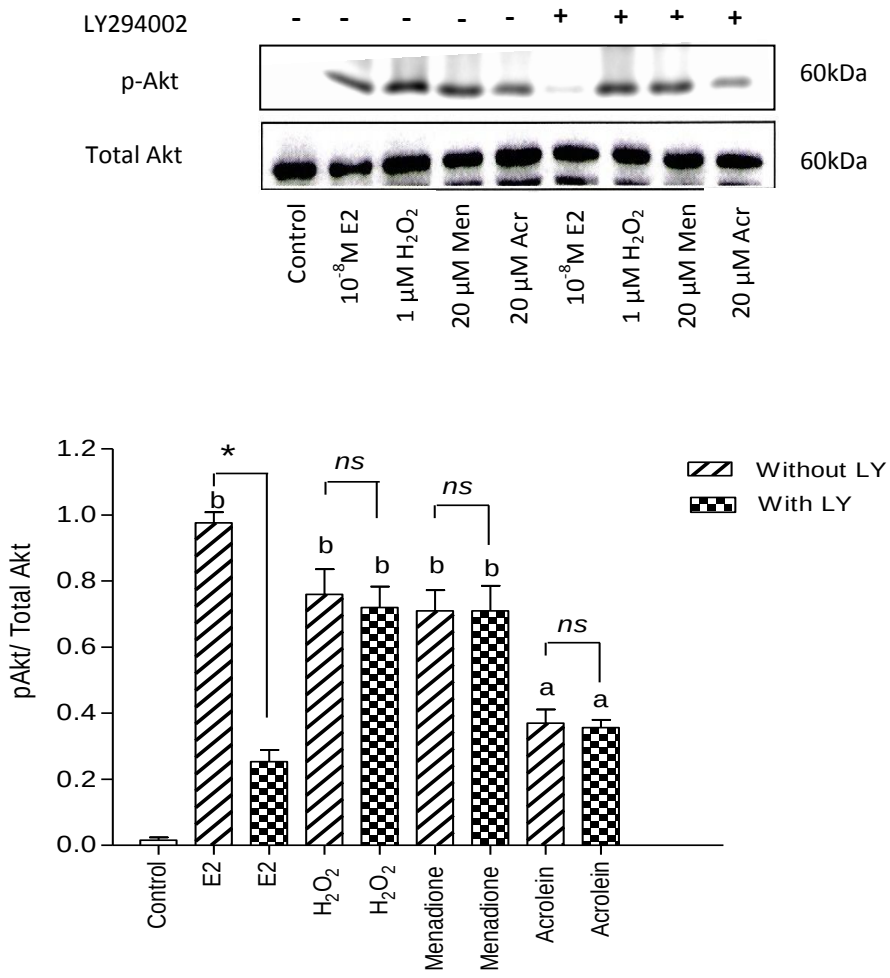
Cells were pre-treated with or without 10 µM LY294002 for 1 h followed by addition of 1 µM H_2O_2, 20 µM menadione or 20 µM acrolein for 48 h, and 10^{-8} M E2 for 24 h. *Figure 5.10* shows a significant increase in p-Akt/total Akt ratio in all treated cells. However, LY294002 inhibited the phosphorylation of E2 treated cells only.

5.3.2.4 Effects of LY294002 on PI3K/Akt activation in response to combination of E2 and oxidants or acrolein

Cells were pre-treated with 10^{-8} M E2 for 24 h followed by addition of 1 µM H_2O_2, 20 µM menadione or 20 µM acrolein for 48 h. To determine the effects of inhibitor, cells were exposed to LY294002 for 1 h before or after addition of 10^{-8} M E2.

Figure 5.11a demonstrates that E2 alone or in combination with oxidants or acrolein significantly induced p-Akt as compared to control. However, the p-Akt/total Akt ratio was not significantly different between E2 and its combination with oxidants or acrolein. The inhibitory effect of LY294002 was only observed when cells were treated with E2 alone. However, there was no significant difference in the effects of LY294002 when this inhibitor was added before (*Figure 5.11b*) or after the addition of E2 (*Figure 5.11c*). It is possible that the induction of phosphorylation by oxidants or acrolein masked the inhibitory effects on E2. It is suggested that E2 induced cell proliferation also involved PI3K/Akt signaling pathway. However, the involvement of PI3K/ Akt signaling pathway in oxidants- or acrolein- induced cell proliferation cannot be excluded as other kinase/s may phosphorylate Akt.

Figure 5.10: Effects of LY294002 on the E2, oxidants or acrolein induced phosphorylation of p-Akt



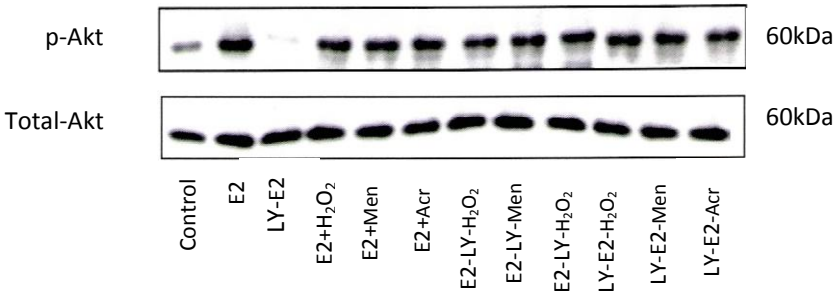
Cells were pre-treated with and without 10 µM LY294002 for 1 h followed by addition of 10^{-8} M E2 for 24 h, 1 µM H_2O_2, 20 µM menadione or 20 µM acrolein for 48 h. The expression of p-Akt and total Akt was determined by Western blotting using Phospho-Akt (Ser473) antibodies and Akt as a loading control. The protein bands were quantified by image J. Values represent mean ± SEM (n=3).

[a] $p<0.05$, [b] $p<0.001$ vs. Control

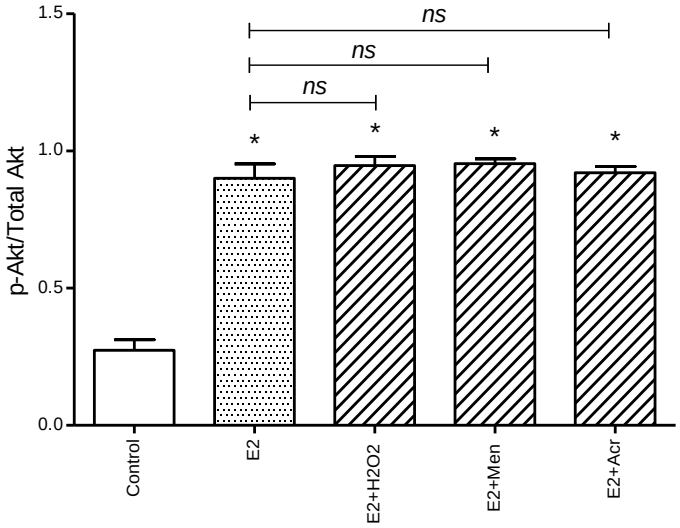
* $p<0.001$ E2 alone vs. E2 with LY

ns: not significant

Figure 5.11: Effects of LY294002 on E2 alone or combination of E2 and oxidants or acrolein induced phosphorylation of PI3K/Akt



a)

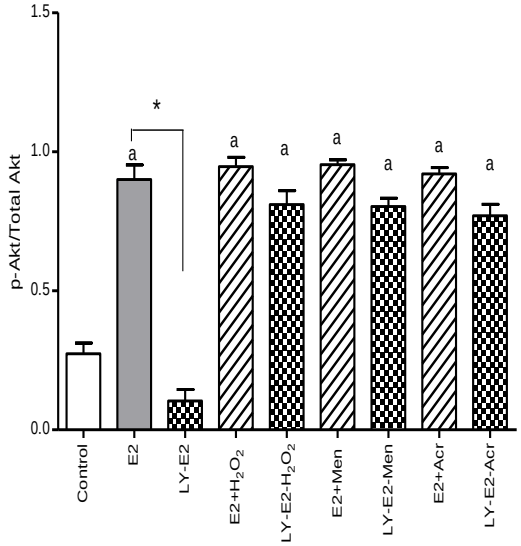


Cells were treated with 10^{-8} M E2 alone for 24 h or combination with 1 µM H_2O_2, 20 µM menadione or 20 µM acrolein for 48 h. The expression of p-Akt and total Akt was determined by Western blotting using Phospho-Akt (Ser473) antibodies and Akt as a loading control. The protein bands were quantified by image J. Values represent mean ± SEM (n=3).

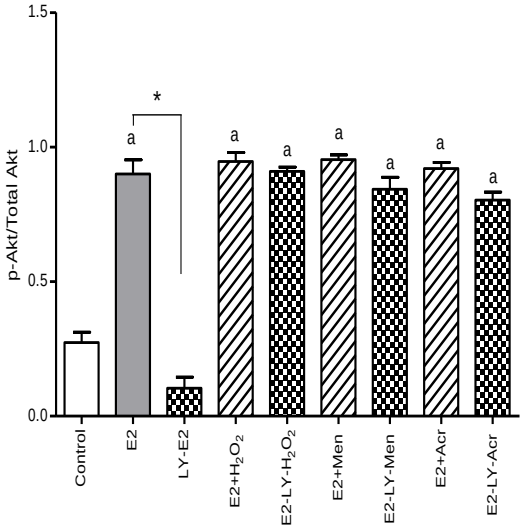
* $p<0.05$ vs. Control

ns: not significant

b)



c)



Cells were treated with 10^{-8} M E2 alone for 24 h or combination with 1 µM H_2O_2, 20 µM menadione or 20 µM acrolein for 48 h. Cells were also exposed to 10 µM LY294002 for 1 h before (b) or after (c) addition of 10^{-8} M E2. The expression of p-Akt and total Akt was determined by Western blotting using Phospho-Akt (Ser473) antibodies and Akt as a loading control. The protein bands were quantified by image J. Values represent mean ± SEM (n=3).

[a] $p<0.001$ vs. Control

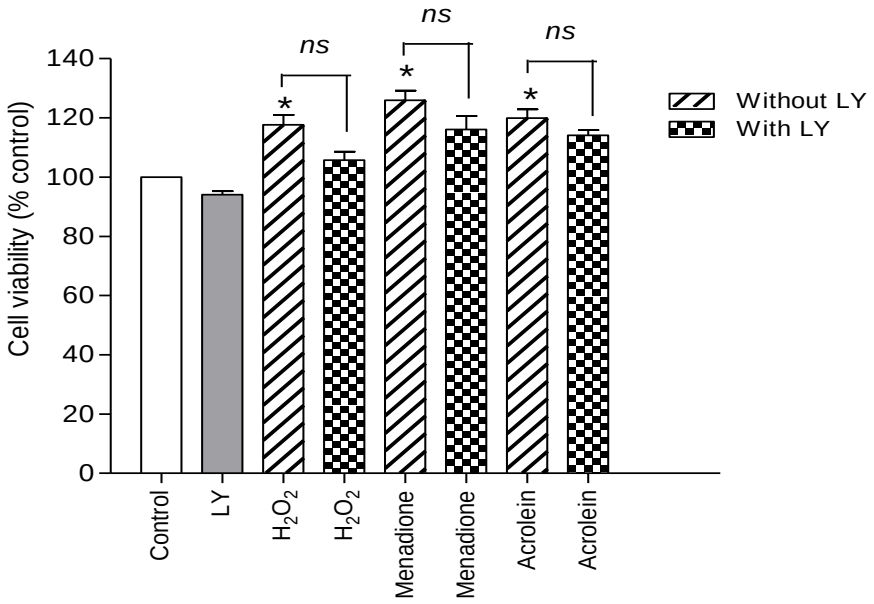
* $p<0.001$ E2 alone vs. E2 with LY

Addition of inhibitor before E2: LY-E2-oxidants or acrolein
Addition of inhibitor after E2: E2-LY-oxidants or acrolein

5.3.2.5 Effects of LY294002 on oxidants and acrolein induced cell proliferation

To further determine whether PI3K/Akt signaling is involved in the mechanism of 12-z endometriotic cell proliferation, cell viability assay was done. Western blotting analysis revealed oxidants, acrolein and E2 induced phosphorylation of p-Akt. However, LY294002 inhibited the E2 treated cells only. In this experiment, cells were pre-treated with or without 10 µM LY294002 for 1 h followed by addition of 1 µM H_2O_2, 20 µM menadione and 20 µM acrolein for 48 h. As shown in *Figure 5.12*, a significant increase of cell viability was observed with the percentages were 114%, 126%, 117% for H_2O_2, menadione and acrolein respectively as compared to control. In contrast to the presence of PD98059, the observed cell proliferation was not inhibited by LY294002. The results indicate that the phosphorylation of Akt by oxidants or acrolein may involve other kinase/s.

Figure 5.12: Effects of LY294002 on oxidants and acrolein induced cell proliferation



Cells were pre-treated with and without 10 µM LY294002 for 1 h followed by addition of 1 µM H_2O_2, 20 µM menadione or 20 µM acrolein for 48 h at the concentrations shown. Cell viability was measured using the MTT assay. Values represent mean ± SEM (n=6) and are expressed as percentage of control (untreated) cells.

* $p<0.05$ vs. Control

ns: not significant

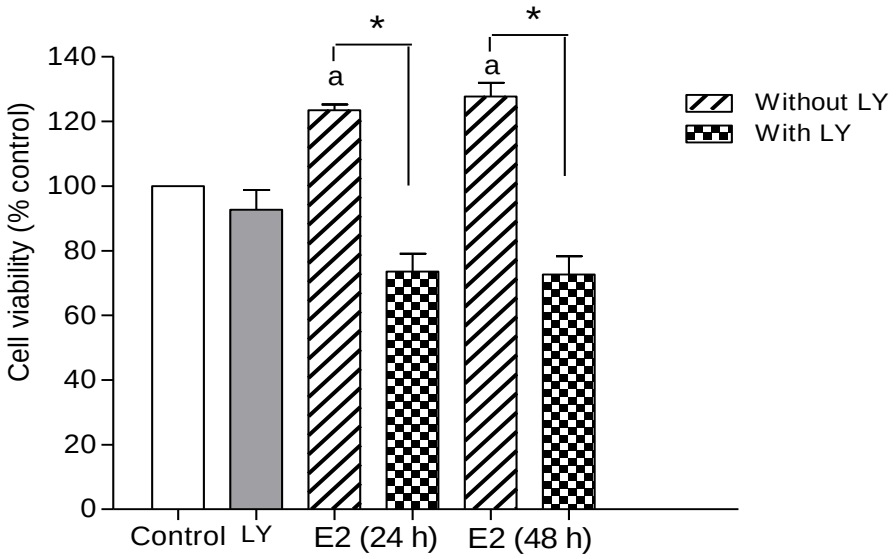
5.3.2.6 Effects of LY294002 on E2 induced cell proliferation

To further determine whether E2 mediates the regulation of PI3K/Akt pathway in 12-z cells, MTT assay was done. Cells were treated with E2 at 10^{-8} M alone for 24 h and 48 h with or without pre-treatment with 10 µM LY294002 for 1 h. As shown in *Figure 5.13*, 10^{-8} M E2 significantly increased cell proliferation, with cell viability of 122% and 128% at 24 h and 48 h respectively as compared to control. A significant reduction of cell growth was observed when cells were pre-treated with inhibitor even cells were allow to grow for 48 h. The percentages of cell viability were 73% and 72% in cells treated with E2 for 24 h and 48 h respectively.

5.3.2.7 Effects of LY294002 on combination of E2 and oxidants or acrolein induced cell proliferation

Cells were pre-treated with 10^{-8} M E2 for 24 h followed by addition of 1 µM H_2O_2, 20 µM menadione or 20 µM acrolein for 48 h. Cells were also exposed to 10 µM LY294002 for 1 h before or after addition of 10^{-8} M E2. Combination of both E2 and oxidants or acrolein significantly increased cell proliferation with the percentages of cell viability were 122%, 123% and 120% for E2 with H_2O_2, E2 with menadione or E2 with acrolein respectively, as shown in *Figure 5.14a, b and c*. There was no cell proliferation observed when LY294002 was added at the beginning. The MTT assay results of inhibitor studies shows E2 is involved in cell proliferation by activating PI3K/Akt pathway as well as the MAPK/ERK1/2 pathway.

Figure 5.13: Effects of LY294002 on E2 induced cell proliferation



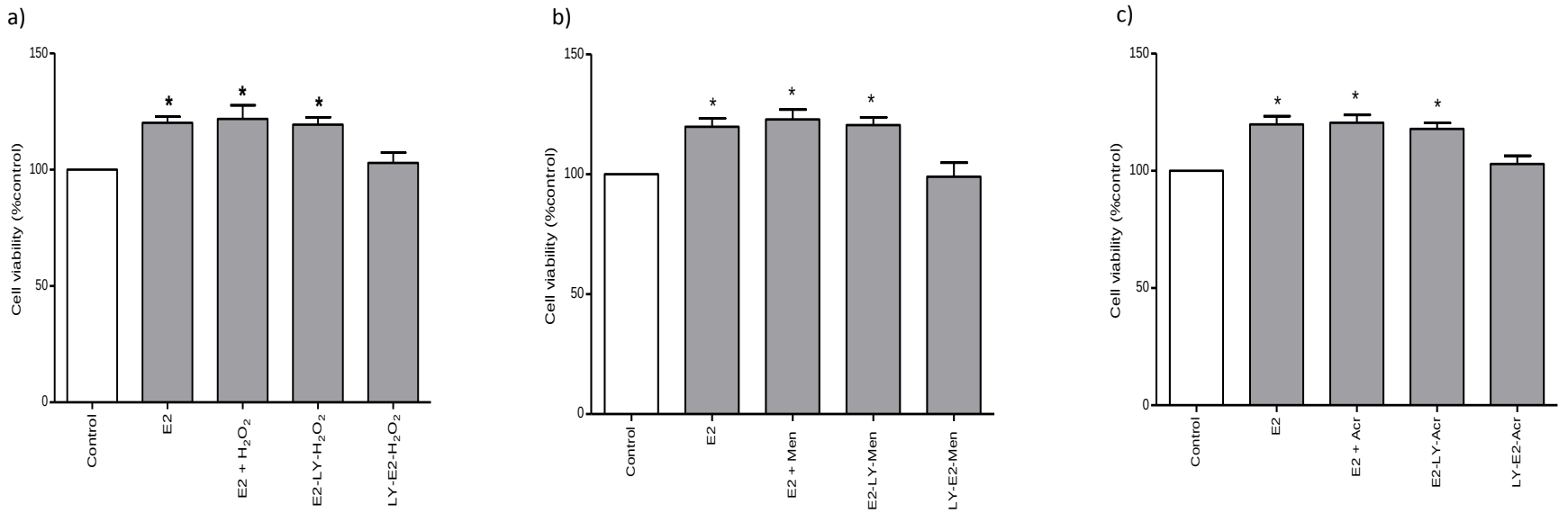
Cells were pre-treated with and without 10 µM LY294002 for 1 h followed by addition of E2 at 10^{-8} M for 24 h and 48 h. Cell viability was measured using the MTT assay. Values represent mean ± SEM (n=6) and are expressed as percentage of control (untreated) cells.

[a] $p<0.05$ vs. Control

* $p<0.001$ E2 alone vs. E2 with LY

CHAPTER 5

Figure 5.14: Effects of LY294002 on E2 alone and E2 with oxidants or acrolein induced cell proliferation



Cells were treated with 10^{-8} M E2 alone for 24 h or combination with a) 1 µM H_2O_2 b) 20 µM menadione or c) 20 µM acrolein for 48 h. Cells were also exposed to 10 µM LY294002 for 1 h before or after addition of 10^{-8} M E2. Cell viability was measured using the MTT assay. Values represent mean ± SEM (n=6) and are expressed as percentage of control (untreated) cells.
*p<0.05 vs Control

Addition of inhibitor before E2:	LY-E2-oxidants or acrolein
Addition of inhibitor after E2:	E2-LY-oxidants or acrolein

5.4 Discussion

The changes in gene expression of estrogen biosynthesis and metabolising enzymes linked to oxidants, acrolein and E2 have been demonstrated earlier in Chapter 4. To further understand the pathogenesis of endometriosis, the present chapter describes the potential role of two signaling pathways, MAPK/ERK1/2 and PI3K/Akt in the mechanism of endometriotic cell proliferation.

5.4.1 Roles of MAPK/ERK1/2 signaling pathway in the mechanism of endometriotic cell proliferation

5.4.1.1 E2 and oxidants activate MAPK/ERK1/2 signaling pathway in endometriotic cells

The ERK pathway was reported as the first MAPK pathway to be identified (Krauss, 2003). The effects of MAPK/ERK1/2 pathway activation depend on cell type and stimulus, however, it is agreed that this pathway is mainly involved in the regulation of cell proliferation, survival and differentiation (Raman *et al.*, 2007). In this chapter, the use of ERK1/2 specific inhibitor, PD98059 suggest E2 and ROS including H_2O_2 and menadione induced cell proliferation through MAPK/ERK1/2 signaling pathway. In addition, the up-regulation of pERK1/2 was much higher in the combination of E2 and H_2O_2 or menadione. These results are consistent with previous studies that found H_2O_2 stimulates ERK1/2 phosphorylation in endometriotic stromal cell (Yoshino *et al.*, 2004). The results of this chapter provides the first evidence that E2 and oxidants induced phosphorylation of ERK1/2 in 12-z cells whereas previous studies showed the induction of this signaling pathway in 12-z cells involved cytokines (Grund *et al.*, 2008).

This chapter provides another evidence of E2 action through the non ERE-dependent pathway. E2 is known to trigger rapidly a variety of second-messenger signaling events including the stimulation of cAMP, calcium mobilization, generation of inositol phosphate and activation of MAPKs (Seval *et al.*, 2006). These authors proposed the rapid signaling events are initiated at the plasma membrane or cytoplasm and do not involve ERE-mediated gene transcription.

Previous studies using Western blotting have demonstrated that E2 alone rapidly increased the ratio of phospho- to total ERK1/2 significantly in endometrium from women with endometriosis compared to endometrium from normal women (Murk *et al.*, 2008). In that study, both types of cells were incubated with E2 for 10 min and then subjected for Western blotting. To further validate the role of ERK1/2 in the cell viability, normal endometrial stromal cells were treated with PD98059 at 20 µM for 24 h and a significant, 50% decreased in cell viability was noted as compared to normal cells without inhibitor.

5.4.1.2 The possible mechanism of endometriotic cell proliferation involves MAPK/ERK1/2

ERK1 and ERK2 have 83% amino acid identity and both are positive regulators of cell proliferation (Lefloch *et al.*, 2008). In endometriosis, there are many possible mechanisms regarding the regulation of cell viability through this pathway. As mentioned earlier, factors that have been reported to induce ERK1/2 phosphorylation include pro-inflammatory cytokine, IL-1β and TNF-α (Yoshino *et al.*, 2004). Wren and co-workers have implicated ERK1/2 in regulating the expression of c-*fos* in endometrial tissue (Wren *et al.*, 2007). *c-fos* is an early transcription factor that is well known to be related to estradiol-dependent cell proliferation. Interestingly, previous studies have also demonstrated that eutopic endometrium from women with endometriosis contained less expression of genes associated with

inactivation of MAPK signaling cascades compared to normal women, which include ERBB receptor feedback inhibitor 1 (ERRFI1), a negative regulator of MAPK signaling (Burney *et al.*, 2007).

Previous studies using endometrial stromal cells and the endometrial epithelial cell line (YHES) has shown that Gonadotropin releasing hormone (GnRH) and transforming growth factor (TGF)-β increased the level of phophorylated ERK1/2 (Luo *et al.*, 2004). GnRH is responsible for the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) whereas TGF-β is known to regulate various cellular activities, including cell growth and differentiation, apoptosis, inflammatory and immune response.

Velarde and colleagues described another possible mechanism of cell proliferation in endometriosis; however human endometrial stromal fibroblasts (hESF) were used in that study (Velarde *et al.*, 2009). These authors demonstrated a significant increase in p-ERK1/2 in hESF from women with endometriosis as compared to normal women, which results in impaired cAMP regulation of cyclin D1 (CCND1) mRNA in endometriosis. CCND1 is critical for early checkpoint regulation at the G1 phase of the cell cycle. cAMP is known to inhibit proliferation in several cell lines by repressing CCND1, thus a downregulation of CCND1 is important for cAMP inhibition of cell-cycle progression. These findings suggest that increased activation of ERK1/2 may be responsible for persistent proliferative effects in endometriosis. Unfortunately, the published data on cAMP regulation of CCND1 is very limited.

5.4.2 Roles of PI3 Kinase/Akt signaling pathway in the mechanism of endometriotic cell proliferation

5.4.2.1 E2 activates PI3 Kinase/Akt signaling pathway in endometriotic cells

Besides activating MAPK/ERK1/2 signaling pathway, this chapter clearly demonstrated that E2 also induced p-Akt phosphorylation similar to previous studies (Zhang *et al.*, 2010). This study also provides the first evidence that E2, oxidants and acrolein induced phosphorylation of Akt in 12-z cells whereas previous studies showed the induction of this signaling pathway in 12-z cells involved cytokines (Grund *et al.*, 2008). Guzeloglu and colleagues showed that E2-mediated phosphorylation of Akt can be observed within 5-15 min in human endometrial stromal cells (Guzeloglu Kayisli *et al.*, 2004). In that study, cells were treated with or without E2 at 10^{-8} M for short (5 min-90 min) and long (3 h-24 h), and time dependent activation of Akt was observed. The results revealed E2 treated cells induced a significant increase in p-Akt at 15 min as compared to untreated cells (control). However, p-Akt level in E2 treated cells returned to baseline levels observed in control cells at 90 min. These authors proposed that long-term E2 treatment of endometrial stromal cells did not affect p-Akt and Akt levels. Thus, it is proposed that rapid E2 action seems to involve non ERE-dependent pathway rather that the classical ERE-dependent pathway as the activation of ERE takes longer time (Guzeloglu Kayisli *et al.*, 2004).

5.4.2.2 Induction of Akt phophorylation by other kinase/s

In contrast to the effects of PD98059, the results of MTT assays revealed the PI3K inhibitor (LY294002) did not blocked H_2O_2, menadione and acrolein induced cell proliferation. In addition, Western blotting has shown that the Akt phosphorylation was not inhibited by LY294002, a selective inhibitor of PI3K. This implicating the involvement of other kinase/s that may phophorylate Akt despite PI3K.

One of the possible kinases is innate immune-signaling kinase, TANK [TRAF (tumour-necrosis-factor-receptor-associated factor)-associated nuclear factor κB activator]-binding kinase 1 (TBK1). TBK1 has been defined as a principle hub in cell regulatory networks responsive to inflammatory cytokines and pathogen surveillance receptors (Fitzgerald *et al.*, 2003). Some of the known functions of TBK-1 are related to the development of the innate antiviral response (Harris *et al.*, 2006) and mediate proliferative activity in endothelial cells (Korherr *et al.*, 2006). Although this kinase has not been examined in endometriotic cells, TBK1 has been shown directly activates Akt by phosphorylation upon mitogen stimulation that trigger the innate immune response in cancer cells (Ou *et al.*, 2011). These authors have determined TBK1 directly activates Akt by phosphorylation of the canonical activation loop (T308) and hydrophobic motif sites (S473) independently of PDK1. Previous authors have reported that TBK-1 is one of the potential targets of ROS (Kolls, 2006) that may support the involvement of TBK-1 induced Akt in this study.

5.4.2.3 Increased ATP production by ROS and acrolein in endometriotic cells

Endometriotic cells share important similarities with neoplastic processes including excessive cell growth (Matalliotakis *et al.*, 2007). Cancer cells must adapt their metabolism to produce adequate energy to promote cell growth and proliferation (Solaini *et al.*, 2011). Therefore, cells need to synthesise more ATP through oxidative phosphorylation that consume most of the cellular oxygen. Similarly, endometriotic cells also need adequate ATP to fulfill the requirement of increasing in cell proliferation. As mentioned earlier, LY294002 is a competitive inhibitor for ATP binding site of PI3K. An increase in ATP production may prevent the inhibitor from binding to the PI3K. Thus, PI3K was not completely inhibited and the phosphorylation of Akt was not abolished. A question remains to be answered, if this mechanism occur, an increase in ATP production in E2 treated cells should be obtained. However, LY294002 was able to completely inhibit PI3K in E2 treated

cells. There is another PI3K inhibitor that commonly used, Wortmannin, a fungal metabolite. In contrast to LY294002, this inhibitor is not ATP competitive inhibitor. The use of this inhibitor may be considered in future.

In summary, the *in vitro* studies in this chapter indicate that both MAPK/ERK1/2 and PI3K/Akt are involved in the mechanism of endometriotic cell proliferation. Using the ERK1/2 inhibitor (PD98059) and PI3K inhibitor (LY294002), it is concluded that oxidants, including H_2O_2 and menadione activates MAPK/ERK1/2 whereas E2 strikingly activates both MAPK/ERK1/2 and PI3K/Akt signaling pathways. Thus, in addition to ERE-dependent pathway, this chapter proposed the involvement of MAPK/ERK1/2 and PI3K/Akt signaling pathways in the mechanism of endometriotic cell proliferation, particularly in 12-z cells. The possible pathways that induce endometriotic cell proliferation are shown in Figure 5.15. Although acrolein has been shown to promote cell proliferation, the signaling pathway/s that involves this compound needs further investigation. The identification of pathways involved in the pathogenesis of endometriosis provides new insight of potential targets for therapeutics to inhibit disease progression.

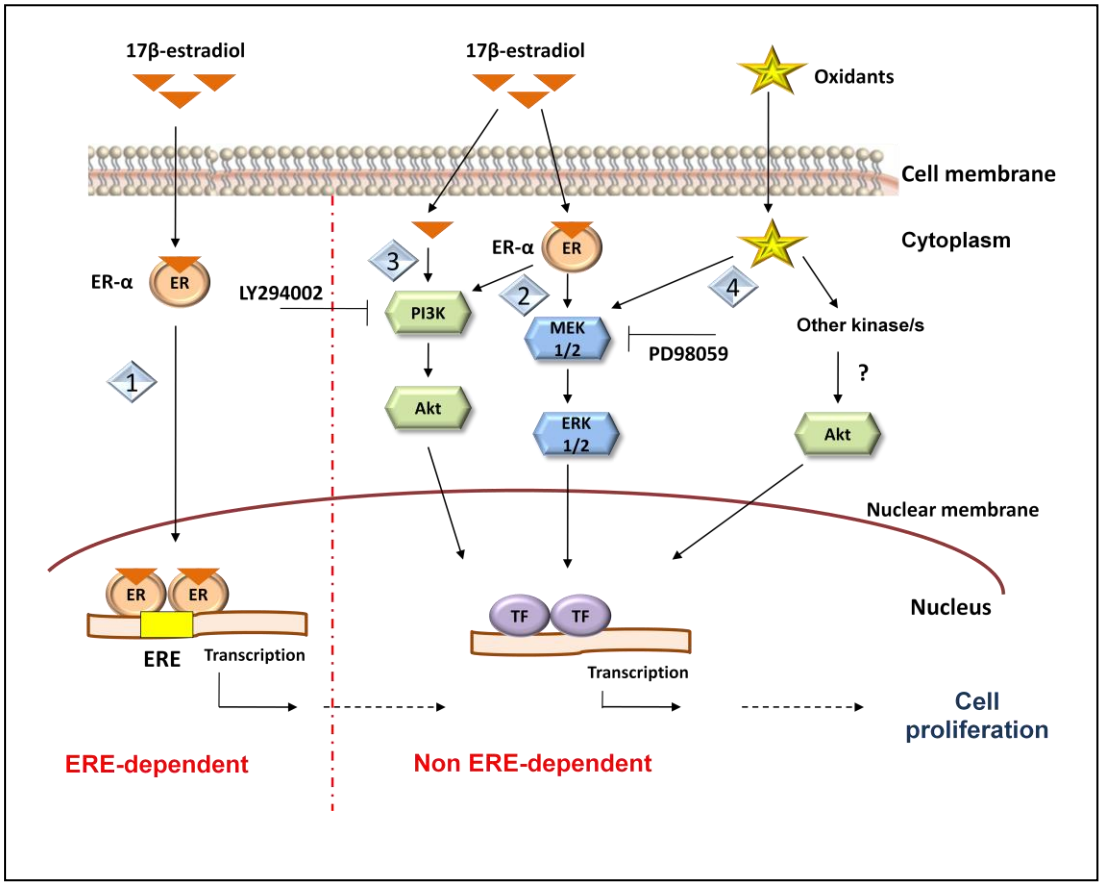


Figure 5.15: A proposed mechanism of cell proliferation that involves signaling pathways in 12-z cells.

ERE-dependent pathway (Pathway 1): Ligand-activated ER-α bind specifically to EREs in the promoter of target genes leading to the induction of gene transcription. **Non ERE-dependent pathway; Pathway 2**: Ligand-bound ER-α activate the ERK1/2 MAPK and PI3K/Akt signal transduction pathways. **Pathway 3**: E2 directly activates PI3K/Akt pathway without ERs.
Pathway 4: Oxidants directly activate the ERK1/2 MAPK pathway. The activation of Akt may also involve other kinase/s. *TF, transcription factor.*

CHAPTER SIX:

ANTI-PROLIFERATIVE EFFECT OF THE ANTIOXIDANT N-ACETYLCYSTEINE IN ENDOMETRIOTIC CELLS

6.1 Introduction

Increased ROS levels and depletion of antioxidants have been suggested to contribute to excessive proliferation of endometrial stromal cells in patients with endometriosis (Foyouzi *et al.*, 2004). Cells can normally deal with a low level of oxidants through the presence of antioxidant molecules (Halliwell, 1994). An antioxidant has been defined as any substance that delays, prevents or removes oxidative damage (Halliwell, 2007). Antioxidants can be divided into enzymatic and non-enzymatic. Examples of enzymatic antioxidants include catalase, superoxide dismutase (SOD), glutathione reductase, glutathione transferase, glutathione peroxidase (GPx) (Pocernich *et al.*, 2001) and heme-oxygenase (HO)-1 (Van Langendonckt *et al.*, 2002c) whereas non-enzymatic include N-acetylcysteine (NAC), ascorbic acid (vitamin C), α-tocopherol (vitamin E), glutathione (GSH), carotenoids and flavonoids (Valko *et al.*, 2007), selenium, zinc, taurine (Agarwal *et al.*, 2005), bilirubin, biliverdin and ferririn (Ryter *et al.*, 2006). In addition, antioxidants can also be classified as endogenous or exogenous. Endogenous are made by the body such as GSH, SOD and catalase, on the other hand, exogenous are coming from the diet or supplements such as vitamins and NAC. However, only the non-enzymatic antioxidants are discussed in this chapter.

6.1.1 Roles of endogenous non-enzymatic antioxidants in cell protection

The most abundant endogenous antioxidant is glutathione (GSH) which is present in millimolar concentrations in the cytosol of cells (Valko *et al.*, 2007). Dickinson and colleagues have reported that GSH is critically important for detoxifying reactive substances including xenobiotics i.e chemicals foreign to biological systems, peroxide compounds and other free radical-generating molecules (Dickinson *et al.*, 2003). Thus, it exerts profound protective effects on cells. It has three components of amino acids; glutamate, glycine and cysteine in which the sulfhydryl (–SH) of the

cysteine is involved in the reduction and conjugation reactions (Forman *et al.*, 2009). GSH is well accepted as the major intracellular low-molecular-weight thiol (sulfhydryl (–SH) compound that plays a pivotal role in the cellular defense against elevated ROS by maintaining the cellular redox status (Sato *et al.*, 2009; Wang *et al.*, 2011). Redox status refers to the ability of the cells to cycle between oxidised and reduced forms (Forman *et al.*, 2009). In mammals, ROS that are generated through respiration are scavenged in the mitochondria by GSH, meaning it has an essential function in mitochondria under normal physiological condition (Izawa *et al.*, 1995). The protective effect of GSH is based on generating its oxidised form, glutathione disulfide (GSSG). In brief, GSH that has free thiol group provides reducing equivalents for the glutathione peroxidase (GPx) catalysed reduction of H_2O_2 to water. In this process GSH becomes GSSG that is converted back to its GSH form by glutathione reductase (GR) using β-nicotinamide adenine dinucleotide phosphate (NADPH) to maintain a high cellular GSH/GSSG ratio (Dickinson & Forman, 2002). Therefore, when mammalian cells are exposed to increase ROS, the ratio of GSH/GSSG will decrease. This mechanism is shown in *Figure 6.1*.

Various diseases have been associated with elevated ROS and/or a low cellular GSH level such as atherosclerosis (Rosenblat *et al.*, 2002), diabetes (Shurtz-Swirski *et al.*, 2001) and neurodegeneration (Seyfried *et al.*, 2000). It can be concluded that glutathione protects the cells from elevated ROS, which either cause oxidative damage or increase in cell proliferation in endometriosis. Thus, it is important to maintain the intracellular GSH level in order to prevent ROS effects. Unfortunately, there is no available data regarding the association of GSH with endometriosis.

6.1.2 Roles of exogenous non-enzymatic antioxidants NAC in cell protection

Exogenous antioxidants also play an important role against oxidative damage by ROS. NAC is one of the known exogenous antioxidants that is widely used to counter the adverse effects of elevated ROS (Maheshwari *et al.*, 2011). It has also

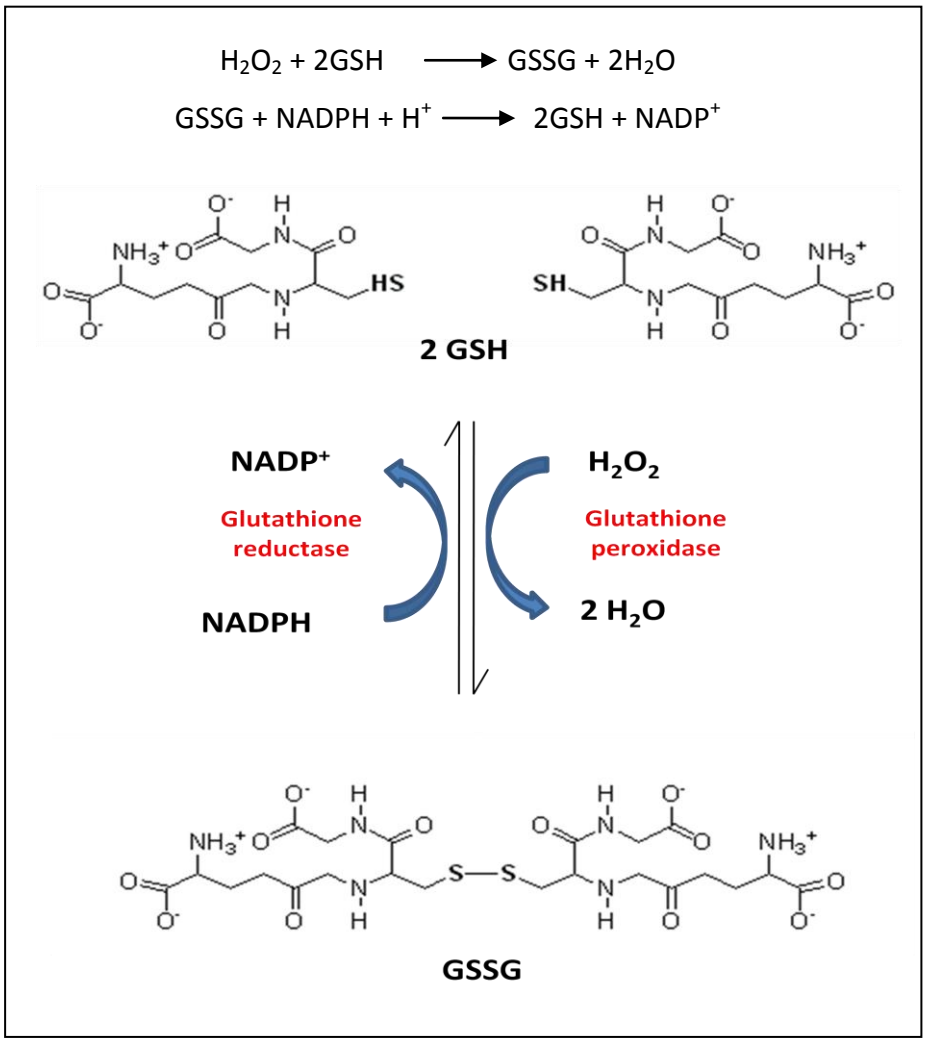


Figure 6.1: Mechanism of ROS removal by glutathione. In the presence of ROS (H_2O_2), reduced GSH is converted to GSSG by glutathione peroxidase. Conversion back to GSH in the cell is catalyzed by NADPH and glutathione reductase.

been utilized as a tool for investigating numerous biological and pathological processes associated with elevated ROS (Zafarullah *et al.*, 2003). Our hypothesis is that elevated ROS leads to cell proliferation in endometriosis. Hence the role of this antioxidant as an antiproliferative agent is the main focus of this chapter.

Cysteine is a sulfur-containing non-essential amino acid that can be synthesised by the body. It is also commonly found in high-protein meat products. NAC is a synthetic cysteine derivative, in which the nitrogen atom of the amino group is attached to an acetyl group (Sato *et al.*, 2009). Chemically, NAC is similar to cysteine except it has an acetyl group. The effectiveness of NAC is known due to its ability to act intracellularly as a source of sulfhydryl group whereas the acetyl group provides the stability of the NAC molecule and reduce the reactivity of the thiol as compared to cysteine. The sulfhydryl group of NAC stimulates GSH synthesis, enhances glutathione-S-transferase activity and is capable of scavenging free radicals (De Vries & De Flora, 1993). In comparison to cysteine, NAC is less toxic, less susceptible to oxidation and is more soluble in water (Bonanomi & Gazzaniga, 1980). The chemical structures of cysteine and NAC are shown in *Figure 6.2*.

6.1.2.1 Antioxidant effect: NAC scavenges ROS

The well known function of NAC is as an antioxidant, either directly related to the drug itself by scavenging ROS (Aruoma *et al.*, 1989) or to the secondary induction of glutathione production (Karlsson *et al.*, 2011). The hydrogen atom in the sulfhydryl (−SH) group of many sulfur-containing anti-oxidant molecules (thiols) can act as an electron for neutralising free-radicals. Free radicals that scavenged by NAC are hydroxyl radicals (·OH) and superoxide anion ($O2\cdot^{-}$) (Aruoma *et al.*, 1989). The rate constants of the reaction of NAC to scavenge ROS is lower than the enzymatic antioxidants including SOD, catalase and glutathione peroxidase (Jones *et al.*, 2003), which makes the role of NAC in free radical scavenging *in vivo* less important.

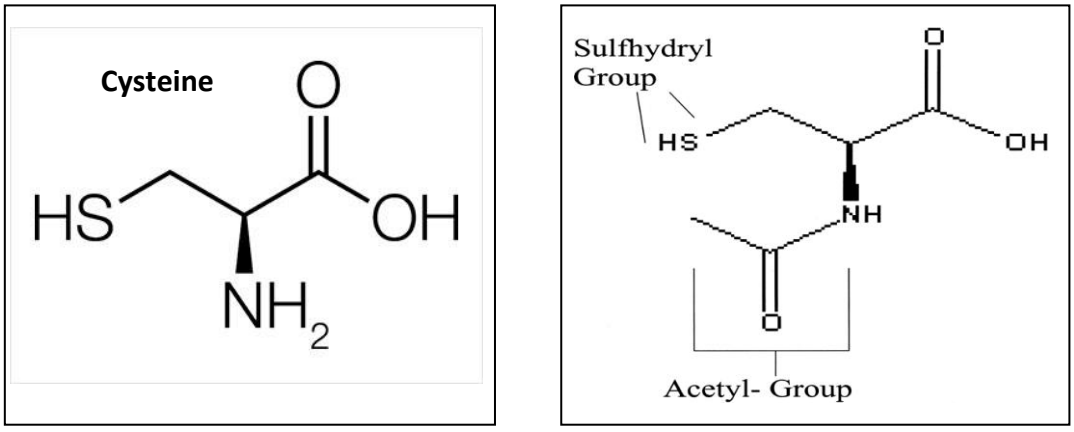


Figure 6.2: Chemical structures of cysteine and N-acetylcysteine. The presence of acetyl-group in NAC differentiates this molecule from cysteine. The potent reactivity of NAC is due to the sulfhydryl group whereas the acetyl group provides its stability.

6.1.2.2 Antioxidant effect: NAC increases intracellular GSH levels

The balance between the antioxidant defence and oxidation production in cells is important to prevent oxidative damage. The intracellular redox environment must be more reducing than being oxidative to maintain cell function at an optimal level (Atkuri *et al.*, 2007). Some of the major inter-dependent redox couples including GSH/GSSG, NADPH/$NADP^+$ and NADH/NAD^+. Among the three components amino acids, cysteine has the lowest intracellular concentration (Dickinson *et al.*, 2003). Furthermore, the bio-availability of cysteine inside the cell has been found as a common limiting factor in GSH synthesis and 90% of cysteine in the extracellular compartment is in oxidised form (Kinscherf *et al.*, 1994). Since cysteine *per se* is very unstable in its reduced form (Sen, 1998), numerous studies have been conducted that focused on alternative strategies for cysteine delivery. NAC is very stable, easily enters the cell and rapidly deacetylated into cysteine that is readily taken up by cells and converted into GSH (Sen, 1998), which promotes the

glutathione redox cycle, one of the important ROS-removing systems (Zafarullah *et al.*, 2003). Hence, NAC is a precursor for the intracellular synthesis of GSH.

6.1.2.3 NAC has anti-inflammatory properties

Inflammatory cytokines have been implicated in the pathogenesis of endometriosis (Yoshino *et al.*, 2004). Although the association of NAC with cytokines has not been studied in endometriosis, this antioxidant has been proved to reduce IL-6 levels in hemodialysis patients (Nascimento *et al.*, 2010) and TNF-α and IL-1β in rat model of traumatic brain injury (Chen *et al.*, 2008). However, the exact mechanism of reduction in cytokines production by NAC is not well clarified.

6.1.2.4 Roles of NAC in apoptosis

Most of the previously reported *in vitro* studies have demonstrated that NAC counteracts elevated ROS and prevents the induction of apoptosis (Maheshwari *et al.*, 2011). For example, NAC prevented H_2O_2 induced apoptosis through down-regulation of caspase 9 and c-Jun/JNK signalling pathway in male germ cells. Male germ cells that were pre-treated with 5 mM NAC significantly prevented the apoptosis induced by 10µM H_2O_2 measured using TdT-mediated deoxyuridinetriphosphate nick end labelling (TUNEL) assay. In addition, the increased expression of Bax, a pro-apoptotic protein was reversed back to control levels. Thus, it is clear that NAC has anti-apoptotic properties.

Conversely, although most of the published data demonstrated that NAC has anti-apoptotic effects, previous studies using immortalized endometrial stromal cell line (Yale Human Endometrial Stromal or YHES cell line) proposed that NAC induces apoptosis through Fas/FasL signaling pathway (Wu & Guo, 2006). In that study, 1 µM H_2O_2 significantly induced cell proliferation that suppressed by 10 mM NAC. In

the same study, Fas and FasL expression was determined at 8 h, 18 h and 36 h in NAC treated cells at 10 mM and compared with untreated cells. FasL is known to initiate apoptotic signals upon binding to its receptor. The results revealed significant expression of FasL in NAC treated cells as compared to control at 18 h and 36 h of treatment, which implicates NAC in inducing apoptosis in this cell line.

6.1.3 Chapter Aims

Although emerging evidence indicates that elevated ROS is one of the leading factors in the pathogenesis of endometriosis, several important questions remain unanswered regarding the roles of oxidants and antioxidants in endometriotic cell proliferation. There is still an ongoing debate over the role of antioxidants as a treatment of endometriosis. With evidence implicating the involvement of antioxidants as anti-proliferative, it is essential to identify the mechanism in endometriosis, which may lead to valuable therapeutic approaches for limiting the disease progression.

To achieve this aim, the work in this chapter has the following objectives:

1. To determine the effects of the antioxidant NAC on cell proliferation and cell death
2. To determine the role of apoptosis in mediating these effects. The roles of mitochondrial dependent pathway and c-Jun NH2-terminal kinase (JNK) signaling pathway are going to be examined.
3. To determine the role of NAC in preventing the effects of elevated ROS in endometriotic cells

6.2 Materials and methods

Inhibitor:

c-Jun NH2-terminal kinase (JNK) inhibitor, SP600125 was used in this study to determine the involvement of JNK signaling pathway in the mechansim of apoptosis. Chemical name: [Anthra (1, 9-cd) pyrazol-6(2H)-one; 1, 9-Pyrazoloanthrone]. The inhibitor was purchased from Sigma, UK.

The solution was prepared as below:

10 mg of SP600125 (MW=220.23) was dissolved in 227.3 µl DMSO to prepare the stock solution (20 mM). An amount of 10 µl of 20 mM SP600125 was made up to 20 ml with culture media to prepare 10 µM SP600125 working solution.

All other materials and methods for this chapter are described in chapter 2.

6.3 Results

Previous chapters have determined that several oxidants induce 12-z cell proliferation. This chapter aims to discover whether antioxidants have anti-proliferative effects in endometriotic cells. The involvement of different apoptotic pathways as a possible cause of growth inhibition has also been investigated.

6.3.1 Effects of NAC on cell growth and viability

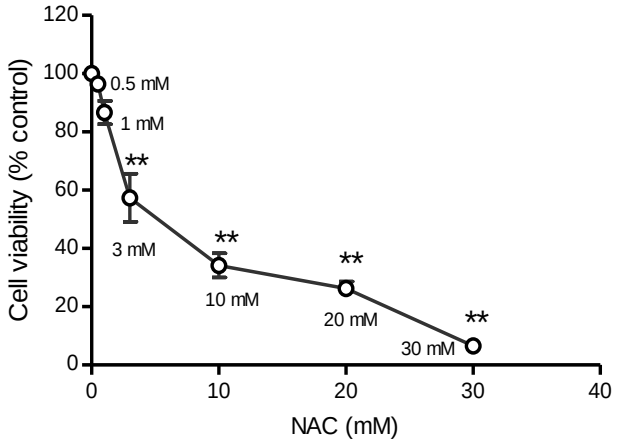
To determine the effects of NAC on cell growth and viability, cells were treated with 0.5 mM-30 mM NAC for 24 h and cell viability was evaluated using the MTT assay as described previously. *Figure 6.3a* demonstrates there is no increase in cell proliferation observed at any of NAC concentrations used. NAC was observed to significantly inhibit cell growth at 3 mM-30 mM.

6.3.2 Effects of NAC on cell membrane integrity

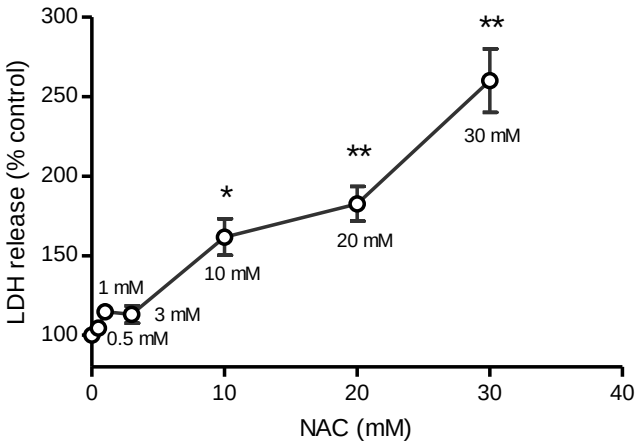
To determine effects of NAC on cell membrane integrity, cells were treated with 0.5 mM-30 mM NAC for 24 h and lactate dehydrogenase (LDH) leakage was determined as described previously. As shown in *Figure 6.3b*, LDH leakage was significanty observed when cells were exposed to 10 mM-30 mM NAC. Therefore, these higher concentrations appear to induce irreversible cell death or necrosis. At 3 mM NAC, there was no significant LDH release. Hence it is likely that despite the significant loss of viability at 3 mM that observed in *Figure 6.3a*, this concentration caused apoptotic cell death.

Figure 6.3: Effects of NAC on 12-z cell viability.

a)



b)



Cells were treated NAC at concentrations shown for 24 h. Cell viability was measured by a) MTT assay and b) Lactate dehydrogenase (LDH) assay. Values represent mean ± SEM (n=6) and are expressed as percentage of control (untreated) cells.

* $p<0.01$, ** $p<0.001$ vs. Control.

6.3.3 Mechanism of NAC-induced cell death

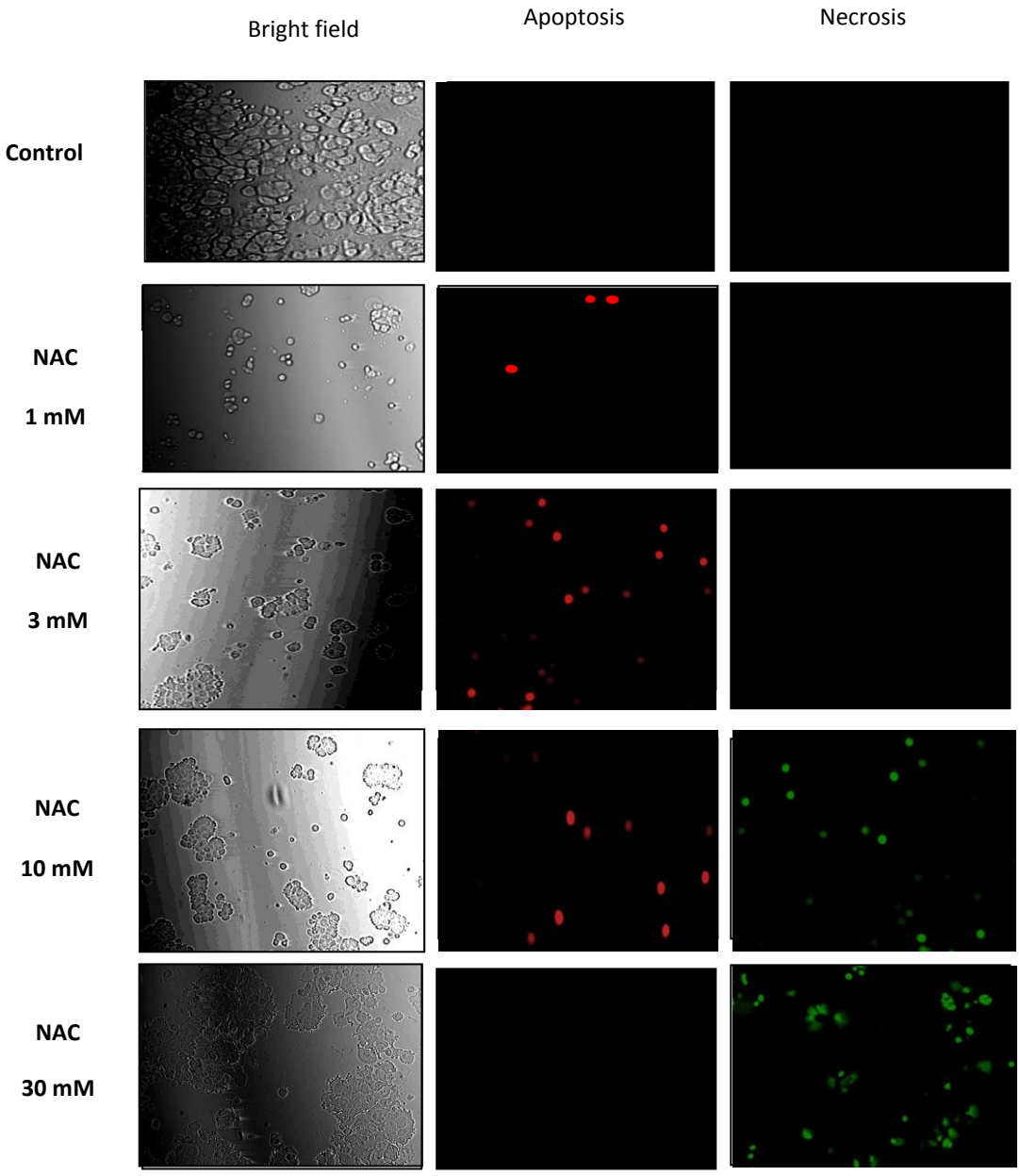
6.3.3.1 NAC induced apoptotic and necrotic cell death

To evaluate the mechanism of cell death induced by NAC, cells were plated in 6 well plates at a concentration of 1 x 10^5 cells/well for 24 h. Cells were then treated with 1 mM-30 mM NAC 24 h. Cells were stained with apoptosis/necrosis specific dye. As shown in *Figure 6.4*, there were no apoptotic or necrotic cells in the control (untreated group). NAC at 1 mM induces very minimal apoptosis and without necrosis. At 3 mM, most of the cells became apoptotic. Cells started to become necrotic at 10 mM where there were presence of both apoptotic and necrotic cells. At high concentration (30 mM), all the cells become necrotic. As suspected, at 3 mM NAC, the majority of cells are apoptotic, and NAC is clearly able to induce apoptotic pathways.

6.3.3.2 Effects of NAC on indicators of apoptosis

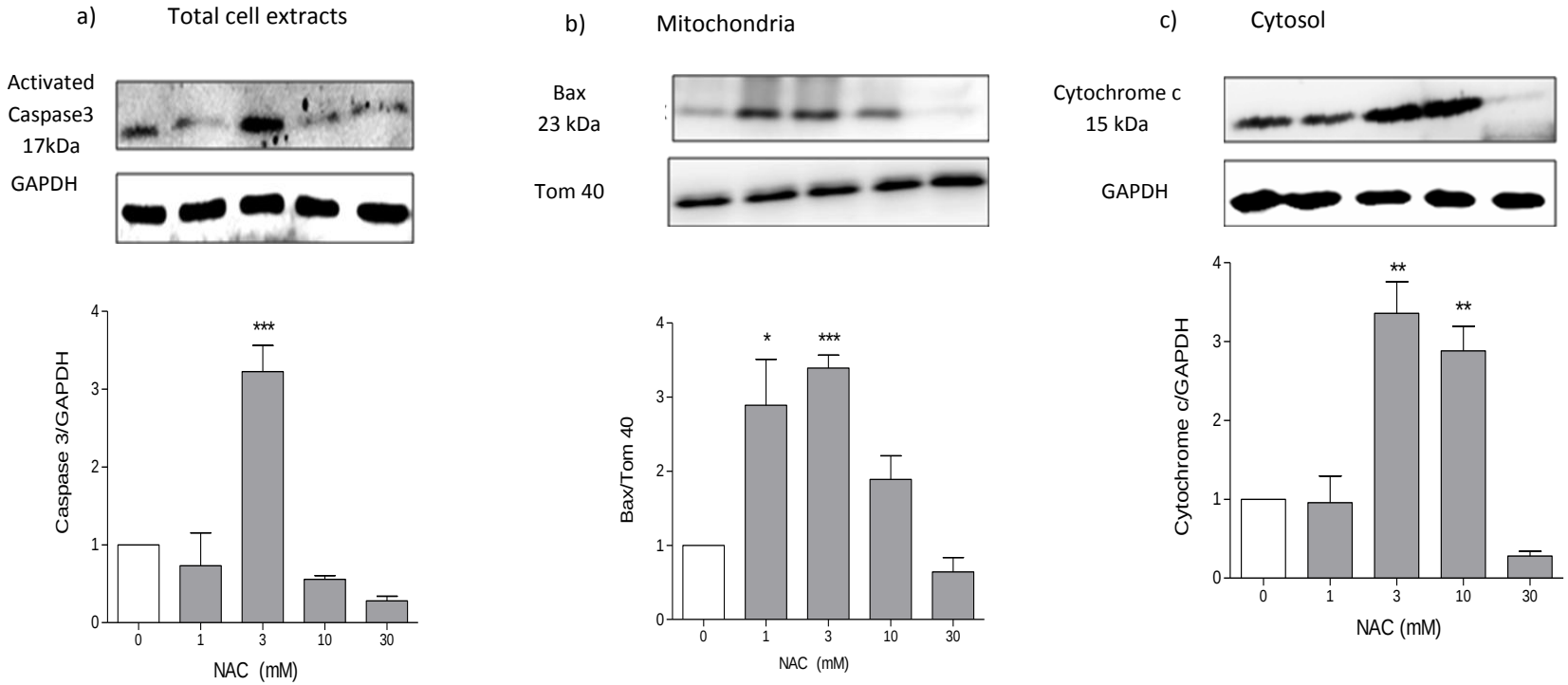
To evaluate whether the NAC induced apoptosis observed in 12-z cells involves the mitochondrial dependent pathway, cells were treated with NAC at 1 mM-30 mM for 24 h and the levels and location of apoptotic indicators (activated caspase3, Bax: pro-apoptotic and cytochrome c) were determined by Western blotting in cell extracts and subcellular preparations. Activated caspase 3 was significantly increased at 3 mM NAC, but decreased at higher concentrations (*Figure 6.5a*). *Figure 6.5b* shows Bax protein levels increased significantly in the mitochondria of cells that were treated with 1 mM and 3 mM NAC. The level of cytosolic cytochrome c was significantly increased by 3 mM and 10 mM NAC as compared to control, as shown in *Figure 6.5c*. Clearly, NAC induces apoptotic marker expression especially at 3 mM. These results support the involvement of the mitochondrial dependent pathway in NAC induced apoptosis in 12-z cells.

Figure 6.4: NAC induced apoptotic and necrotic cell death



NAC induced apoptosis and necrosis was detected in 12-z cells by Fluorescence microscope using AnexinV-Cy3 and SYTOX staining. Cells were treated with NAC at 1 mM, 3 mM, 10 mM and 30 mM for 24 h. Apoptotic cells stained red fluorescence whereas necrotic cells stained green fluorescence.

Figure 6.5: Effects of NAC on indicators of apoptosis in 12-z cells



Graphs show a) Activated caspase 3 (whole cell extracts), b) Bax (mitochondrial fraction) and c) Cytochrome c release (cytosolic fraction) normalised with GAPDH (Activated caspase 3 and cytochrome c) and Tom 40 (Bax) in 12-z cells treated with 1 mM-30 mM NAC for 24 h. Levels of the apoptotic indicators were analysed by Western blotting. The protein bands were quantified by image J. Values represent mean ± SEM (n=3).

* $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs. Control.

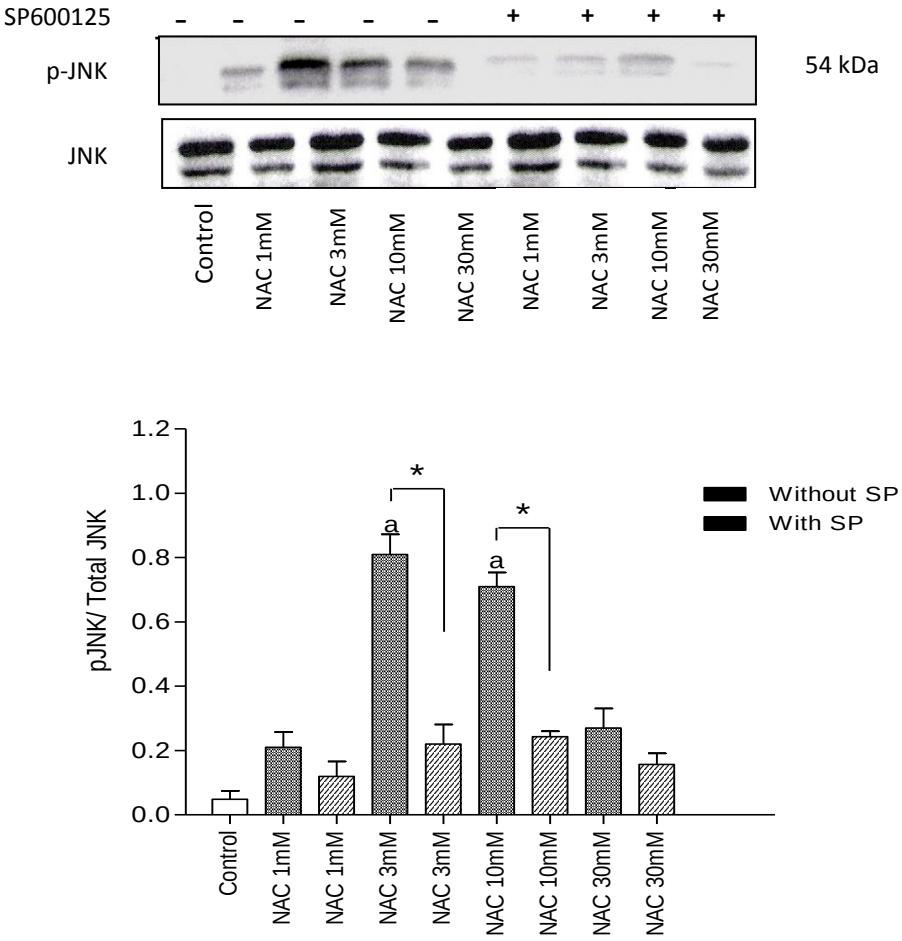
6.3.3.3 Effects of SP600125 on NAC-induced JNK phosphorylation

In order to determine how 3 mM NAC induces apoptosis, the activation of the c-Jun N-terminal kinase (JNK) signaling pathway was examined. The involvement of this pathway in the induction of apoptosis has been widely studied in different types of cells. Cells were treated with 1 mM-30 mM NAC for 24 h and JNK phosphorylation was determined by Western blotting. In addition cells were also pre-treated with SP600125, which is known to inhibit the phosphorylation of JNK, for 1 h before the addition of NAC. As shown in *Figure 6.6*, NAC at 3 mM and 10 mM significantly increased JNK phophorylation as compared to control, and this was prevented when cells were pre-treated with SP600125.

6.3.3.4 Effects of SP600125 on NAC-induced activated caspase 3 and Bax expression

To determine whether activation JNK regulates caspase 3 activation and/or mitochondrial Bax accumulation, cells were pre-treated with or without SP600125 for 1 h followed by addition of 1 mM-30 mM NAC. As shown in *Figure 6.7a*, SP600125 prevented the mitochondrial accumulation of Bax at 1 mM and 3 mM NAC. However, this inhibitor did not prevent the activation of caspase 3 at 3 mM NAC, as shown in *Figure 6.7b*. Thus, it is proposed that JNK may be involved in the mitochondrial dependent mechanism of NAC induced apoptosis. In addition, the results indicate that other apoptotic pathways may be present.

Figure 6.6: Effects of SP600125 on NAC-induced JNK phosphorylation

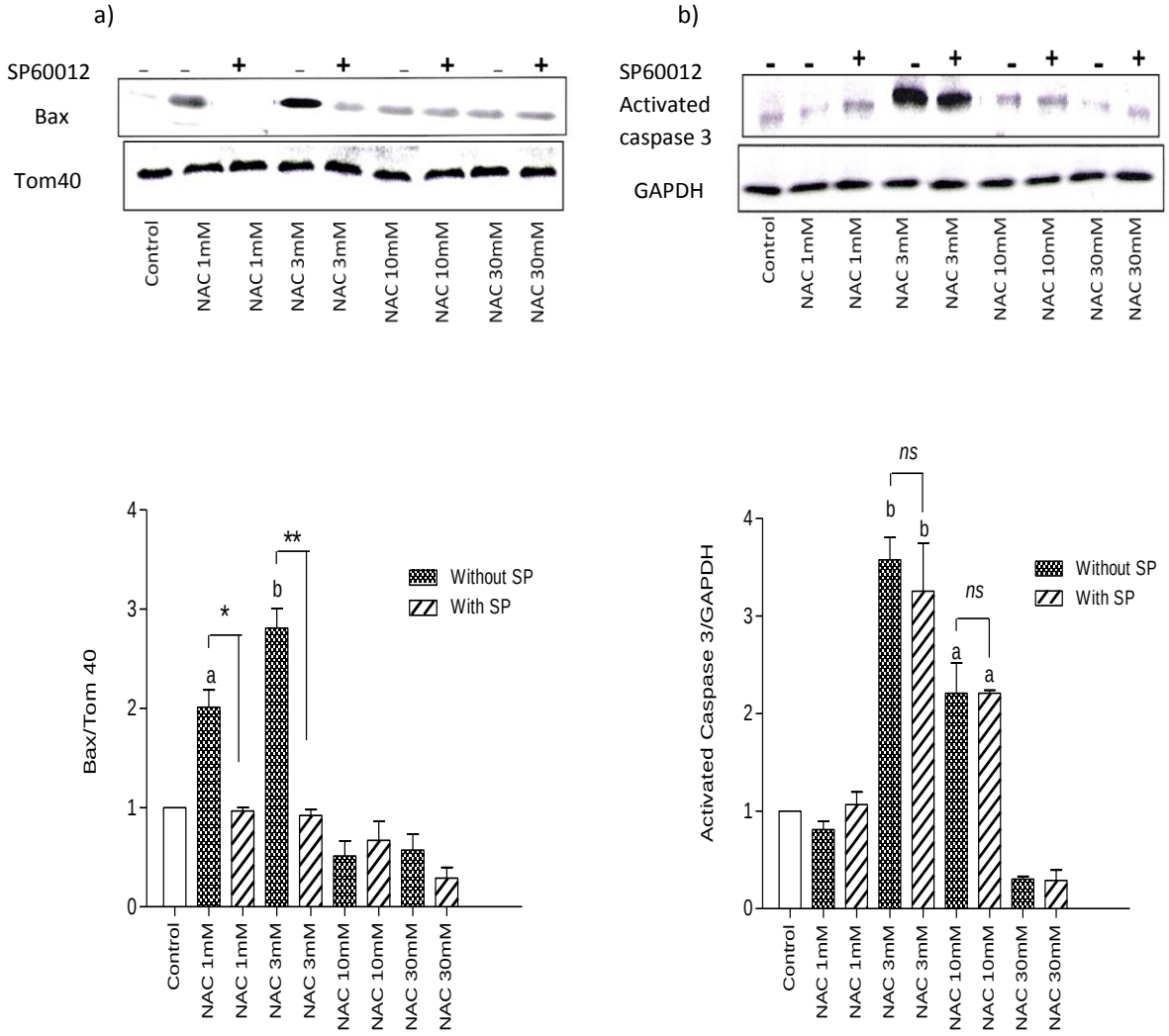


Cells were treated with 1 mM-30 mM NAC for 24 h in the presence and absence of SP600125. Levels of p-JNK were analysed by Western blotting using phospho-JNK antibodies and JNK as a loading control. The protein bands were quantified by image J. Values represent mean ± SEM (n=3).

[a] $p<0.001$ vs. Control

* $p<0.001$ NAC alone vs. NAC with SP

Figure 6.7: Effects of SP600125 on NAC induced activated caspase 3 and Bax expression



Cells were pre- treated with and without SP600125 for 1 h followed by addition of 1 mM-30 mM NAC for 24 h. The expression of a) Bax (mitochondrial fraction) and b) Activated caspase 3 (total cell extracts) were determined by Western blotting. The protein bands were quantified by image J. Values represent mean ± SEM (n=3).
[a]$p<0.01$, [b]$p<0.001$ vs. Control
* $p<0.01$, ** $p<0.001$ NAC alone vs. NAC with SP
ns: not significant

6.3.4 Effects of NAC on H_2O_2 induced proliferation

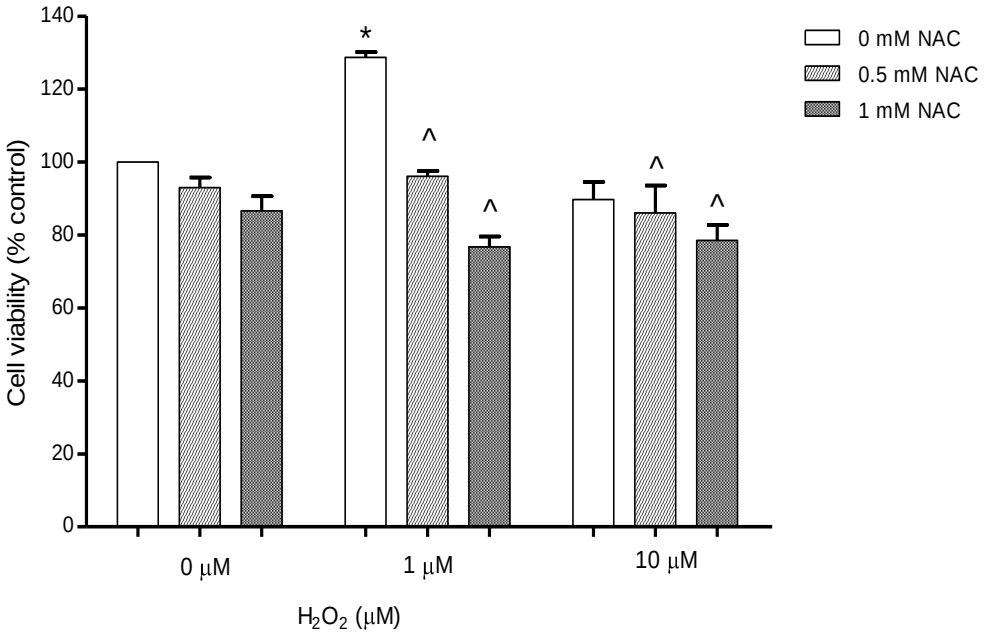
The previous works has shown that NAC on its own cause apoptosis to 12-z cells. To determine whether NAC has anti-proliferative effects on H_2O_2 induced cell proliferation, cells were pre-treated with 0.5 mM-1 mM NAC for 24 h. Then, H_2O_2 at 1 µM (shown previously to induce cell proliferation) or 10 µM was added and cells cultured for another 48 h. The higher concentration of H_2O_2 was also used to determine whether the increase in H_2O_2 may overcome any inhibitory effects of NAC. *Figure 6.8* shows as expected that treatment of 12-z cells with 1 µM H_2O_2 alone significantly induced cell proliferation where cell viability was 129% of control. However, when cells were pre-treated with NAC at 0.5 mM or 1 mM followed by addition of 1 µM or 10 µM H_2O_2, a significant suppression of cell growth was noted as compared to cells that were treated with 1 µM H_2O_2 alone. Thus, NAC significantly suppresses H_2O_2 induced cell proliferation even when the H_2O_2 concentration was increased to 10 µM. It is possible that NAC has enhanced antioxidant status of the cell that prevents H_2O_2 induced cell proliferation. Only 0.5 mM and 1 mM NAC were used in this experiment as NAC at 3 mM and above caused cell death in 12-z cells.

6.3.5 Effects of NAC on glutathione levels

To determine whether NAC at low concentrations may improve the glutathione redox balance, total glutathione levels were measured. Cells were treated with 0.5 mM-3 mM NAC for 24 h and followed by addition of 1 µM H_2O_2 for 48 h. *Figure 6.9a* shows that adding 0.5 mM – 1 mM NAC significantly increased levels of total GSH, but not at 3 mM NAC. However, the levels of total GSH were lowered by the presence of 1 µM H_2O_2. This was countered by the presence of 0.5 mM and 1 mM NAC, which were able to restore GSH to normal levels as above. NAC at 3 mM was unable to elevate GSH levels, which may be due to the induction of apoptosis at this concentration as previously shown.

In *Figure 6.9b*, there was no significant different in the levels of GSSG in all treated cells as compared to control. Figure 6.9c shows a significant reduction of GSH/GSSG ratio was observed in cells that were pre-treated with 0.5 mM-1 mM NAC followed by addition of H_2O_2 as compared to cells that were treated with NAC only. This indicates glutathione redox balance prevents cells from the effects of elevated ROS.

Figure 6.8: Effects of NAC on H_2O_2-induced proliferation

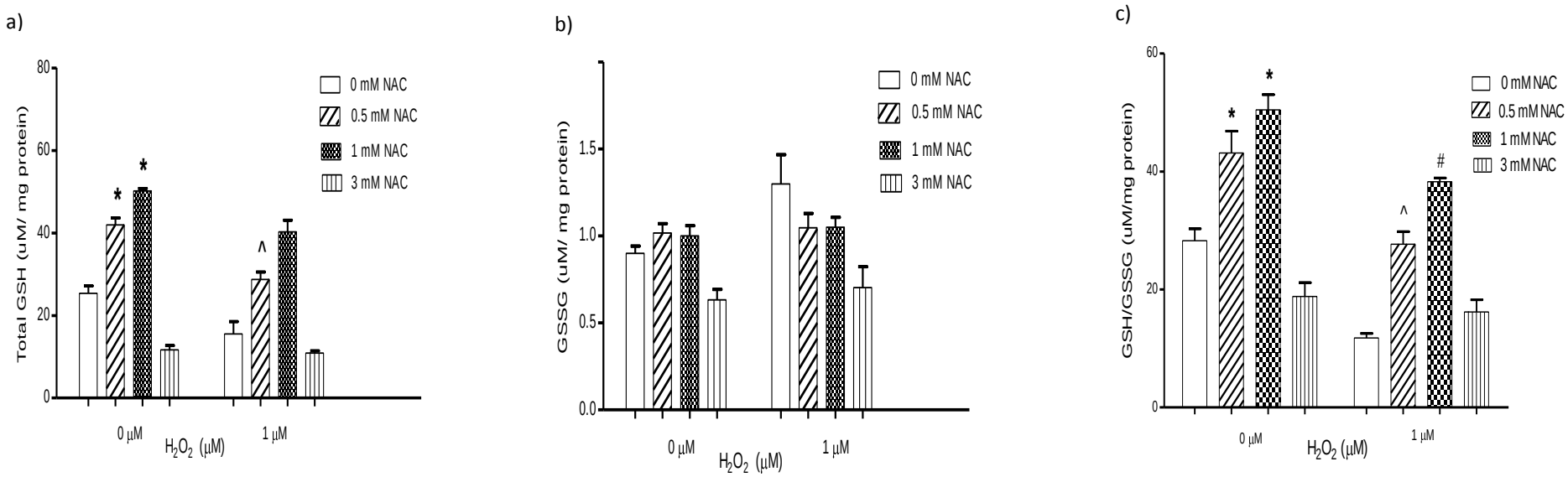


Cells were pre-treated with 0.5 mM and 1 mM NAC for 24 h followed by addition of H_2O_2 at 1 μM or 10 μM for 48 h. Cell viability was measured by MTT assay. Values represent mean ± SEM (n=6) and are expressed as percentage of control (untreated) cells.

* p<0.05 vs. Control

^p<0.001 vs 1 μM H_2O_2 alone

Figure 6.9: Effects of NAC on total glutathione levels



Effects of NAC on glutathione levels in the presence and absence of 1 µM H_2O_2. 12-z cells were pre-treated with 0.5 mM-3 mM NAC for 24 h followed by addition of 1 µM H_2O_2 for 48 h. Levels of a) Total GSH b) GSSG and c) GSH/GSSG ratio were determined with Ellman reagent. Values represent mean ± SEM (n=3) and are expressed as percentage of control (untreated) cells.

* $p<0.05$ vs. Without NAC or H_2O_2

^ $p<0.05$ vs. 0.5 mM NAC alone

# $p<0.05$ vs. 1 mM NAC alone

6.4 Discussion

The main purpose of this chapter was to determine the antiproliferative and antioxidant properties of NAC that inhibit endometriotic cell proliferation and protect cells from the effects of elevated ROS.

6.4.1 Mechanism of cell growth inhibition by NAC

In this study there was no cell proliferation when cells were treated with NAC alone at all concentrations used. The inhibitory effect on 12-z endometriotic cell growth was observed even at low concentration of NAC, 0.5 mM. The inhibitory effect of NAC was confirmed by MTT and LDH assays. The viability assays suggested NAC at 3 mM caused apoptotic cell death; despite no significant release of lactate dehydrogenase in LDH assay, a significant reduction of cell viability was found using the MTT assay. Thus, the findings of this chapter have demonstrated that NAC inhibits cell proliferation, which is consistent with previous studies (Foyouzi *et al.*, 2004; Wu & Guo, 2006; Pittaluga *et al.*, 2010).

Most recently, the effects of NAC on cell proliferation was performed *in vivo* using a murine model of endometriosis (Pittaluga *et al.*, 2010). Endometriosis was induced in 60 female mice by inoculating endometrial fragments into the peritoneal space through a small laparotomy incision. In half of the groups, NAC was administered by gavage, a tube inserted into the stomach through the mouth, for 3 weeks. Several parameters were examined including lesion mass, cell proliferation and differentiation, and the expression of inflammation related genes. A significant weight reduction of endometriomas related to their size was observed in NAC treated cells as compared to controls. Immunohistochemical detection demonstrated that E-cadherin, an inducer of cell differentiation was found diffused in cytoplasm in non-treated NAC whereas in NAC treated mice, E-cadherin staining was only detected along the cell borders. In addition, COX-2 and matrix metalloproteinase (MMP)-9, markers of inflammatory and

invasive respectively, were found to be down-regulated in NAC treated cells by q-RT-PCR. Thus, NAC inhibit cell proliferation by switching cell behavior from proliferation toward differentiation, and by decreasing both tissue inflammation and cell invasiveness.

6.4.1.1 NAC induces apoptosis in 12-z cells

This chapter also provides evidence that NAC inhibits endometriotic cell proliferation by triggering apoptosis in the cells. In this chapter, the involvement of mitochondrial dependent pathway and c-Jun NH2-terminal kinase (JNK) signaling pathway are proposed as the mechanism of NAC-induced apoptosis.

6.4.1.1.1 NAC induces apoptosis involves mitochondrial dependent pathway

Mitochondria are central to both normal cell function and the regulation of cell death. Disruption of the mitochondrial membrane permeabilization (MMP) is one of the earliest intracellular events that occur following the induction of apoptosis (Wang *et al.*, 2011). The mitochondrial pathway is intrinsic and is activated from within the cell (Krauss, 2003). MMP is known to affect the inner and outer mitochondrial membrane to a variable degree where outer membrane permeabilization results in the release of cytochrome c and caspase-independent death effectors including apoptosis-inducing factor (AIF) (Galluzzi *et al.*, 2007). Moreover, these authors have also reported that inner membrane permeabilization results in the disruption mitochondrial ion and volume homeostasis. However, it is still unclear whether the inner membrane permeabilization is the main factor of MMP or as a secondary to outer membrane permeabilization.

The roles of intrinsic and extrinsic pathways in the mechanism of apoptosis have been described in Chapter 1. In brief, intrinsic pathway involves death-promoting stimuli from the cellular compartments that favour MMP wheras the extrinsic pathway

involves the lethal signal comes from the extracellular microenvironment and is transduced within cells (Galluzzi *et al.*, 2007). In this chapter, Western blot analysis revealed the apoptotic indicators caspase 3, Bax and cytochrome c expression were upregulated by NAC especially at 3 mM. The presence of antioxidant NAC in this study promotes the release of cytochrome c from mitochondria that involves Bax. Lethal stimuli may activate pro-apoptotic proteins of the Bcl-2 family including Bax, which may translocate from the cytosol to outer membrane (Wei *et al.*, 2001). Therefore, the conformational changes within the outer mitochondrial membrane by Bax forming a pore-like structure for cytochrome c to escape. The apoptosome formation activates and cleaves the procaspase 3 into caspase 3 leading to apoptotic cell death. Various methods have been recommended to evaluate the MMP including the determination of apoptotic indicators in subcellular fractions by Western blotting (Galluzzi *et al.*, 2007) that used in this study. Other methods to evaluate MMP that proposed by these authors include visualization of ruptures outer membrane of mitochondrial by Electron microscope and localization of inner mitochondrial membrane soluble protein, which can be observed by Fluorescence microscope. From the Western blotting analysis, it is obvious that NAC induced apoptosis through the changes in the MMP.

6.4.1.1.2 NAC induced apoptosis involves the c-Jun N-terminal kinase signaling pathway

The apoptotic program is regulated by extracellular controls, which may activate or suppress the signals. In addition to the extracellular controls, the apoptotic program is also controlled by intracellular signaling pathways (Krauss, 2003) including JNK signaling pathway. JNK pathway represents one sub-group of MAP kinases (Weston & Davis, 2007). Briefly, in this signaling pathway, the JNK is activated by dual phosphorylation on Thr and Tyr by members of the MAPKK group of protein kinases. The phosphorylation of MAPKK is mediated by a group of MAPKKK. Therefore, JNK signaling pathway is mediated by a MAP kinase module: a MAP3K, a MAP2K and a MAPK (Davis, 2000). The series of these protein kinase cascades have been shown

earlier in Chapter 1. Although this signaling pathway is mainly described to be involved in apoptosis, it has also been shown to activate cell proliferation (Zhang & Liu, 2002). A major target of the JNK signaling pathway is the activator protein-1 (AP-1), which is activated mainly by the phosphorylation of c-Jun (Weston & Davis, 2007). This activation subsequently mediates nuclear events leading to cell death (Wang *et al.*, 2007). Compelling evidence has demonstrated that c-Jun plays an important role in cell death under *in vitro* and *in vivo* conditions (Wang *et al.*, 2007). Thus, a blockade of JNK activation prevents cell death. Besides c-Jun, JNK also phosphorylates and regulates the activity of other transcription factors including ATF2, Elk-1, p53 and c-Myc (Liu & Lin, 2005).

JNK plays an important role in post-ischemia/reperfusion cell survival, necrosis, and apoptosis (Wang *et al.*, 2007). Several data shows that JNK can function as a pro-apoptotic kinase (Davis, 2000; Lee *et al.*, 2009). For example, it has been demonstrated that apoptosis induced by nerve growth factor (NGF) withdrawal in rat pheochromocytoma was suppressed by inhibiting the JNK pathway (Liu & Lin, 2005). However, the mechanism by which JNK involves in apoptosis is still controversial. Although numerous studies reported the association of JNK and apoptosis, this signaling pathway has also been implicated to have anti-apoptotic function where it supressed apoptosis in hematopoietic cells via phosphorylation of the pro-apoptotic Bcl-2 family, BAD (Liu & Lin, 2005) and has also been shown to activate cell proliferation in endometriotic cells (Yoshino *et al.*, 2004). Thus, whether JNK acts as pro- or antiapoptotic functions, depending on cell type, nature of the death stimulus, duration of its activation and the activity of other signaling pathways (Liu & Lin, 2005).

It is interesting to note that JNK can activate the mitochondrial dependent pathway (Weston & Davis, 2007). It has been proposed that JNK can phosphorylate and activate several pro-apoptotic protein. The balance of these signals can cause activation of the mitochondrial dependent pathway through the pro-apoptotic proteins Bax and Bak. Bax has also been reported as a JNK substrate, but the site of phosphorylation has not been identified (Weston & Davis, 2007). A pivotal role for

pro-apoptotic proteins Bax and Bak was demonstrated earlier where in $Bax^{-/-}Bak^{-/-}$ compound mutant fibroblasts, activated JNK was unable to cause the release of mitochondrial cytochrome c and apoptosis (Lei *et al.*, 2002). The results of this chapter support JNK in the activation of the mitochondrial dependent apoptotic pathway following NAC treatment. It was demonstrated that 3 mM NAC significantly induced phosphorylation of JNK. Further evaluation of the apoptotic markers expression revealed that SP600125 inhibited Bax protein translocation. Fluorescence analysis that showed apoptotic changes in NAC treated cells especially at 3 mM further support a reduction in cell viability due to NAC is the results of apoptotic induction. Although the function of JNK in apoptosis is complex, the results in this chapter suggesting that the JNK pathway mediates NAC induced apoptosis through Bax activated mitochondrial dependent pathway.

Activation of caspase cascades has been shown to occur in apoptosis. In this study, 3 mM NAC has been shown to induce the cleavage of caspase protein. However, in contrast to Bax protein, the expression of activated caspase 3 did not inhibit by SP600125, thus other signaling pathway/s may be responsible to induce the activation of this apoptotic indicator. Ko and colleagues have determined that activated caspase 3 was also induced by the Fas ligand, which transduces apoptotic signals from the membrane (Ko *et al.*, 2005). NAC induced apoptosis through Fas/FasL signaling pathway in immortalized endometrial stromal cell line (Wu & Guo, 2006) further support the involvement of this pathway in endometriosis. However, the mechanism of NAC-induced FasL expression or activates Fas/FasL system is not well understood. The proposed mechanism of NAC induced apoptosis that involves both mitochondrial dependent and Fas/Fas Ligand dependent pathways is shown in *Figure 6.10*.

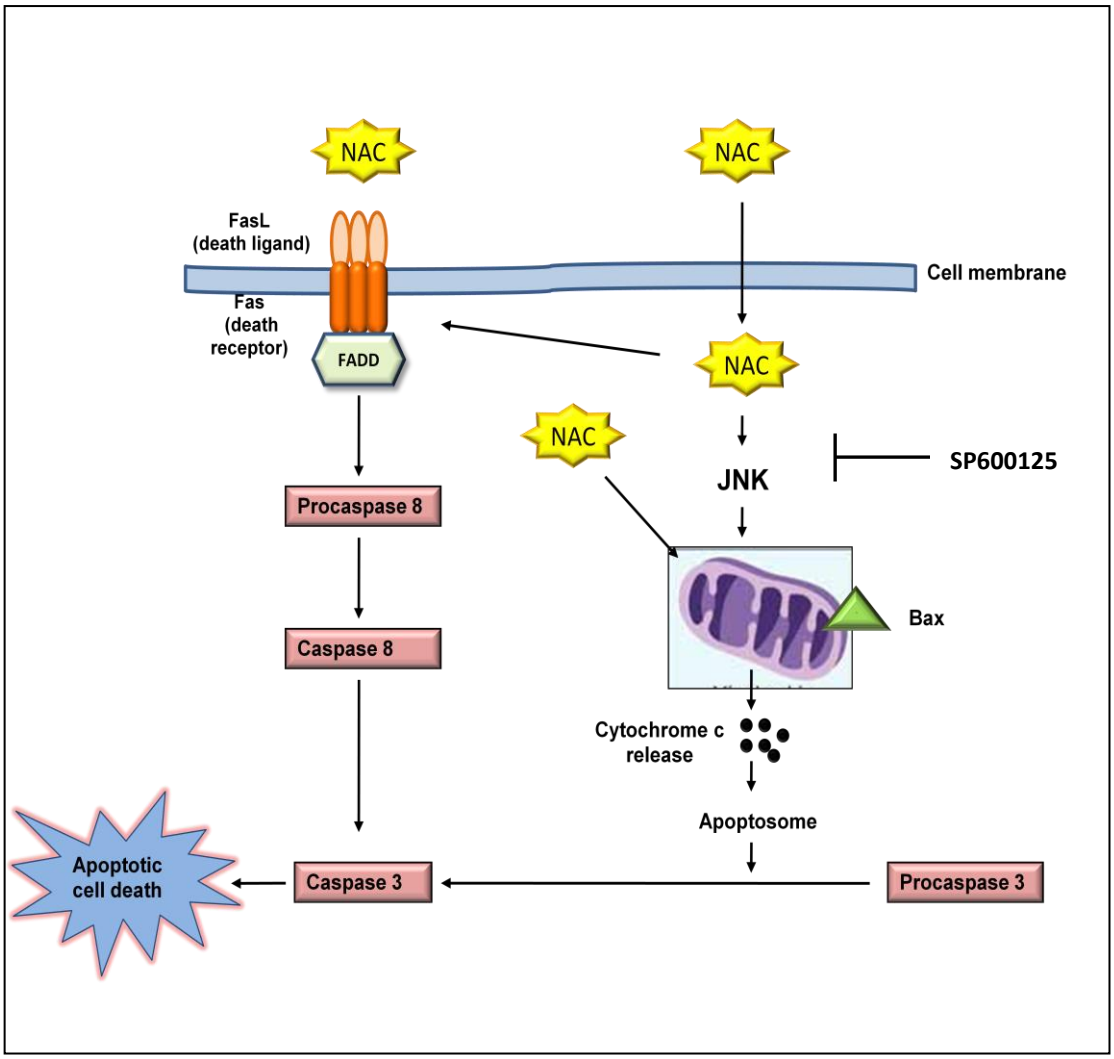


Figure 6.10: Proposed mechanism of NAC induced apoptosis in 12-z cells. NAC induces apoptosis through mitochondrial dependent pathway and JNK pathway through Bax activated mitochondrial dependent pathway. This activation results in release of cytochrome c from mitochondria. The apoptosome formation activates and cleaves procaspase 3 to become caspase 3 and leading to apoptotic cell death. Alternatively, intra- and extracellular NAC activate Fas/FasL system. Fas ligands bind and activate their receptors. Activated receptors recruit the adaptor protein FADD (Fas-Associated Death Domain), which further activate caspase 8 and caspase 3 leading to apoptotic cell death.

6.4.1.2 NAC increases intracellular GSH levels

Glutathione is homeostatically controlled, both intracellular and extracellular where it is being continuously self-adjusting with respect to the balance between GSH synthesis, its recycling from GSSG and its utilisation (Maheshwari *et al.*, 2011). Glutathione is depleted by exogenous stresses including elevated ROS which may explain the low levels of GSH (Berk *et al.*, 2008). In this chapter, when cells were treated with NAC alone at low concentrations, a significant increase GSH levels was observed as compared to the baseline level; however a significant reduction of GSH levels was observed in the presence of 1 μM H_2O_2 that indicate GSH is used to counter H_2O_2. GSH plays a pivotal role in the detoxification process and maintenance of the redox status (Maheshwari *et al.*, 2011). Therefore, it is proposed that being a GSH precursor, NAC-mediated improvement of glutathione redox balance to prevent effects of elevated ROS. Thus, adding NAC increased the amount of the reduced form of glutathione (GSH) in a concentration-dependent manner.

Although there is no available data regarding the association between NAC treatment and GSH levels in endometriosis, previous studies using different types of cells may support the results of this chapter. Most recently, Yu and colleagues have determined Aristolochic acid I (AAI), a botanical product commonly found in herbal remedies induced apoptosis in human promyelocytic leukemia cells (HL-60) and human renal proximal tubular cells (HK-2) (Yu *et al.*, 2011). These authors proposed the apoptotic changes was due to a depletion of intracellular GSH as the GSH level was significantly dropped as compared to untreated cells. Interestingly, the GSH level was significantly improved by the pre-incubation of cells with antioxidant NAC. Thus, NAC that is well known as a clinical antioxidant as well as a GSH precursor was able to replenish AAI-stimulated GSH loss. However, there was no increase in GSH levels when NAC concentration was increased to 3 mM. The cellular change of 12-z

cells by increase concentration of NAC is possible as GSH reduction is one of the signs of apoptotic changes.

6.4.2 N-acetylcysteine as a therapeutic drug

Interest in research on the potential of NAC as a treatment was started many years ago where it was used as a 'mucolytic agent' that is to reduce the viscosity of the mucus in the respiratory tract and later it has been used therapeutically in many diseases related to elevated ROS such as fibrosing alveolitis (Behr *et al.*, 1997), Human immunodeficiency virus (HIV)-positive (De Rosa *et al.*, 2000), prostate cancer (Chiao *et al.*, 2000) and psychiatric disorder, schizophrenia (Berk *et al.*, 2008).

One criteria that has enabled NAC to be approved for clinical use includes its general safety, i.e. is nontoxic to humans (Sen, 1998). The drug has been administered by mainly oral, intravenous routes and via respiratory nebulizer (Fishbane *et al.*, 2004). Following an oral dose, NAC has been reported to be rapidly absorbed into various tissues, deacetylated and metabolised in the intestines and liver.

Peak plasma levels of NAC occur approximately 1 h after an oral dose and become undetectable after 12 h of treatment. Although the bio-availability of NAC is low, 4-10%, oral administration of NAC is still considered clinically effective (Borgstrom *et al.*, 1986). The low bioavailability is due to extensive first pass metabolism by the kidneys and liver (Fishbane *et al.*, 2004). Although NAC is safe to be used, the most common high oral dose of NAC will give side effects such as nausea, vomiting and other gastrointestinal disturbances. However, no clinical adverse reactions have been observed by oral administration of NAC at doses up to 8 g/day (De Rosa *et al.*, 2000).

In summary, it is obvious that NAC inhibits endometriotic cell proliferation at 3 mM. NAC induced apoptosis is proposed as the possible mechanism of cell growth inhibition, which involves mitochondrial dependent and JNK signaling pathways. The antiproliferative effect of NAC may be consistent with the reduced cell proliferation and increased apoptosis. Considerable concern has been expressed since recent years over the best modality of treatment in endometriosis. NAC appears to be promising in the treatment of many diseases. The data in this chapter reinforce the idea that the role of apoptosis in the treatment of endometriosis may be considered and the link between researches in drugs induced apoptosis into clinical application needs further review. Understanding the molecular mechanisms by which NAC inhibits 12-z cell proliferation should provide insights into another alternative treatment and prevention of disease progression.

Although there is very limited published data on the induction of apoptosis by NAC especially in endometriosis, it has been demonstrated that NAC induced apoptosis in an immortalized endometrial stromal cell line through the extrinsic apoptosis pathway, Fas/ Fas L system (Wu & Guo, 2006). In addition, anti-apoptotic Bcl-2 has been studied previously in endometriosis. The Bcl-2 family is the key regulators that control mitochondria-mediated apoptosis that consists of more than 20 members, which can have a negative or positive effect on the initiation of the apoptotic program (Krauss, 2003).

Some drugs that used to control endometriosis act by increasing apoptotic cells (Gomes *et al.*, 2009). For example, the used of combined oral contraceptive for one month significantly reduced cell proliferation and increased the apoptotic index in the eutopic endometrium of patients with endometriosis as compared to without endometriosis (Imai *et al.*, 2000). These authors have proposed the antiproliferative effect was mediated by the activation of the Fas-FasL system. Thus, it remains to be determined whether NAC can provide another potential treatment for endometriosis.

CHAPTER SEVEN:

INDUCTION OF ANTIOXIDANT ENZYMES IN ENDOMETRIOSIS, AND CONTRIBUTION TO PROTECTION AGAINST OXIDANTS AND ACROLEIN

7.1 Introduction

ROS formed as byproducts of mitochondrial oxidative phosphorylation and believed to be a major source of elevated ROS *in vivo* (Mockett *et al*., 1999). Although elevated ROS has been implicated in endometriotic cell proliferation, it is still not clear whether elevated ROS results from increased oxidant production or a failure of intrinsic antioxidant systems. This can include antioxidant molecules or endogenous antioxidant enzymes including haem oxygenase (HO) (Van Langendonckt *et al*., 2002c), catalase, superoxide dismutase (SOD), glutathione reductase, glutathione transferase and glutathione peroxidase (GPx) (Pocernich *et al*., 2001). Of these, haem oxygenase has been given much attention due to its inducible nature. Superoxide dismutase is one of the main constitutive antioxidant defences, and both of HO and SOD enzymes are discussed below.

7.1.1 Haem oxygenase

Haem oxygenase (HO) has been characterised as three isoforms; HO-1, HO-2, and HO-3 (Otterbein & Choi, 2000). HO-1 is highly inducible and HO-2 is constitutively expressed (Quinlan *et al.*, 2008). HO-3 has been demonstrated in rats and to date, its biological function is still not clear (McCoubrey *et al.*, 1997). Different genes encode these, and their expression and regulation differ among cell types (Panahian *et al.*, 1999). The sizes of the protein HO-1, HO-2 and HO-3 are 32-kDa, 36 kDa and 33 kDa respectively (Otterbein & Choi, 2000). HO activity is found in all systemic organs with the highest activity is in spleen, where senescent erythrocytes are sequestered and destroyed.

HO-1 and HO-2 form part of an enzymatic pathway for haem catabolism. They oxidise haem to the bile pigment biliverdin, which requires three moles of molecular oxygen and NADPH. The cleavage of the haem ring results in the concurrent release of free

iron ferrous (Fe^{2+}) and carbon monoxide (CO). Biliverdin is further metabolised to bilirubin by biliverdin reductase (BVR) (Ryter *et al.*, 2006; Kim *et al.*, 2011; Schipper, 2011). Iron released from HO activity triggers the ferritin synthesis (Otterbein & Choi, 2000). In ferritin, iron is stored in the ferric iron (Fe^{3+}) form; however, it must be converted to ferrous before release when the body needs. Ferritin, biliverdin and bilirubin have antioxidant activity wheras CO has a role as an anti-inflammatory (Slebos *et al.*, 2003; Ryter *et al.*, 2006). Haem catabolism pathway is shown in *Figure 7.1*.

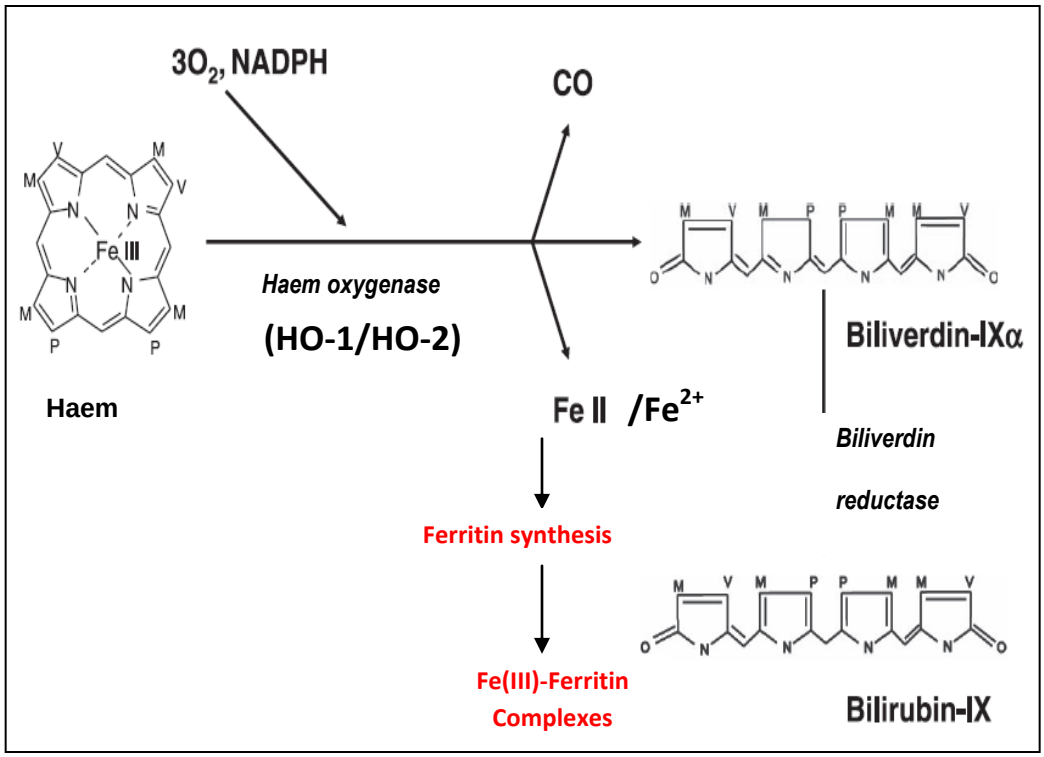


Figure 7.1: Haem catabolism pathway. Haem oxygenase enzymes catalyse haem to the bile pigment biliverdin, which requires three moles of molecular oxygen and NADPH: cytochrome P450 reductase. The cleavage of the haem ring releases free iron (Fe^{2+}) and carbon monoxide (CO). Biliverdin is further metabolised to bilirubin by biliverdin reductase (BVR). Adapted from (Ryter *et al.*, 2006).
M: methyl; V: vinyl; P: propionate

7.1.1.1 Haem oxygenase protects cells from ROS by producing biliverdin, bilirubin and ferritin

Catabolism of hemoglobin results in haem (ferroprotoporphyrin IX) production and its elevation leads to HO induction. Haem at physiological levels plays an important role in various biological reactions including oxygen transport, respiration, drug detoxification and signal transduction (Ponka, 1999). A balance between haem synthesis and catabolism is essential for the maintenance of cellular homeostasis (Dong *et al.*, 2000). The antioxidant protection of HO cannot be explained solely by the release of iron from haem, as iron is also potentially toxic. The antioxidant activity of HO is mainly through the sequestration of free iron by ferritin and the formation of the antioxidants biliverdin and bilirubin (Ryter *et al.*, 2006).

Bilirubin is one of the most abundant endogenous antioxidant in mammalian tissues that account for the majority of the antioxidant activity of human serum (Gopinathan *et al.*, 1994). In mammals, bilirubin at normal concentrations is an efficient scavenger of singlet oxygen and acts as a reducing agent for certain peroxidases including horseradish peroxidase and prostaglandin H synthase (Abraham & Kappas, 2008). Others have reported that at low concentrations, bilirubin is able to scavenge ROS *in vitro*, thus reducing oxidant-mediated cellular damage (Stocker *et al.*, 1987). Bilirubin can scavenge peroxyl radicals as efficiently as α-tocopherol or vitamin E in the brain (Stocker *et al.*, 1987). Bilirubin is rapidly converted from biliverdin by biliverdin reductase (Ryter *et al.*, 2006).

The iron-storage protein ferritin is another antioxidant that is produced *in vivo* due to the release of iron following haem breakdown. When iron is not properly liganded, its ferrous form can react with hydrogen peroxide (produced by mitochondria) to produce the very reactive and damaging hydroxyl radical (OH·) via Fenton reaction, as shown in

Figure 7.2. Irons needs to be sequestered rapidly into ferritin (Otterbein & Choi, 2000) as shown in *Figure 7.1*. Thus, induction of ferritin synthesis and sequestration of iron into ferritin is one of the possible detoxification pathways to limit the generation of free radicals .

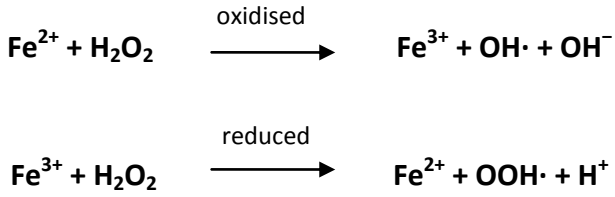


Figure 7.2: Fenton reaction. Ferrous iron (Fe^{2+}) is oxidised by hydrogen peroxide to ferric iron (Fe^{3+}), a hydroxyl radical and a hydroxyl anion. Fe^{3+} is then reduced back to Fe2+ in the presence of hydrogen peroxide to form a peroxide radical and a proton. Adapted from (Waisberg *et al.*, 2003).

7.1.1.2 Roles of haem oxygenase in endometriosis

Cells protect themselves from iron toxicity by expressing HO-1. Excessive hemoglobin causes cytotoxic effects in the peritoneal environment because haem and ferric iron are known as pro-oxidant and pro-inflammatory molecules (Balla *et al.*, 1995). Free haem is known to be toxic to organs, tissues and cells through the generation of ROS (Gutteridge & Smith, 1988; Nhien *et al.*, 2010). The hydroxyl radical produced due to the reaction between ferrous iron and hydrogen peroxide (*Figure 7.2*) can initiate lipid peroxidation (Graca-Souza *et al.*, 2006). Haem induced formation of ROS by the conversion of low reactive organic hydroperoxides (ROOH) that formed during the process of lipid peroxidation is shown in *Figure 7.3*. HO-1 present in the peritoneal mesothelium may help to detoxify the haem produced in endometriosis (Van Langendonckt *et al.*, 2002b). The mechanism of HO-1 activity in endometriosis is shown in *Figure 7.4*.

Lipid peroxidation:

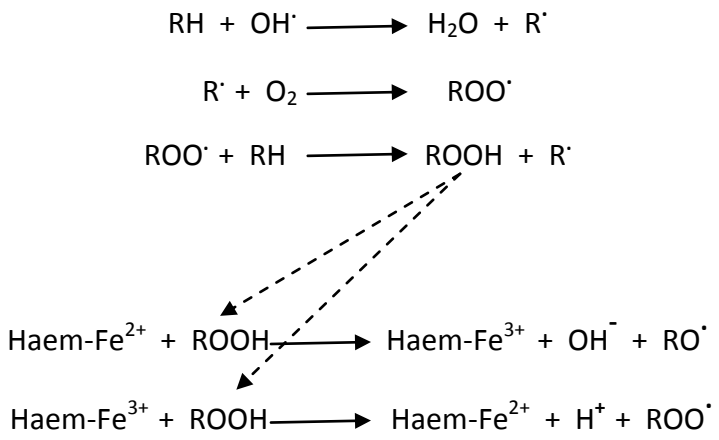


Figure 7.3: The formation of ROS by haem. Haem induced formation of ROS by the conversion of low reactive organic hydroperoxides (ROOH) into highly reactive alkoxyl ($RO^{\bullet}$) and peroxyl ($ROO^{\bullet}$) radicals leading to oxidative stress. Haem complex with iron in the ferrous state is known as ferroprotoporphyrin whereas with the iron in the ferric state is called ferriprotoporphyrin. Adapted from (Graca-Souza *et al.*, 2006).

7.1.1.3 Regulation of HO-1 expression

Among the three isoforms, HO-1 is an inducible protein (Wong & Wispe, 1997). In most tissues that are not directly involved in erythrocytes metabolism, the levels of this enzyme is low to undetectable under basal conditions; however it responds rapidly by various chemical and physical stimuli (Ryter *et al.*, 2006). The expression of HO-1 can be induced by various stimuli including ultraviolet irradiation, endotoxin, heavy metals, and oxidants such as H_2O_2 (Otterbein & Choi, 2000). Other factors that have been reported to induce HO-1 expression include TNF-α in human endothelial cells and IL-6 in mouse macrophages (Ryter *et al.*, 2006). Expression of HO-1 is regulated essentially at the transcriptional level by the redox sensitive transcription factor, nuclear factor E2-related factor 2 (Nrf2) (Slebos *et al.*, 2003). Nrf2 controls the expression and also

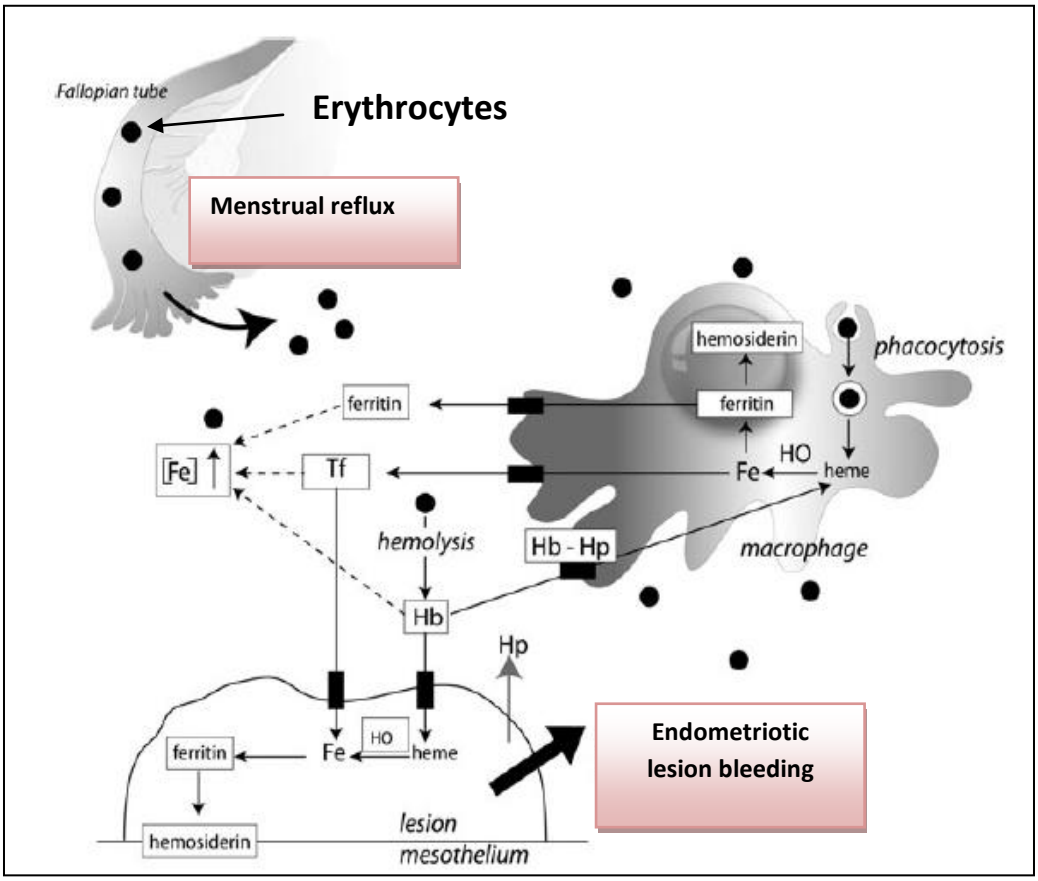


Figure 7.4: Functional consequences of haem oxygenase-1 activity in endometriosis. Hemoglobin released from lysis of erythrocytes and endometriotic lesion bleeding. Degradation of hemoglobin produces biologically active molecule, haem. In the presence of HO-1, haem is catalysed to form free iron (Fe^{2+}). Free iron is sequestered rapidly into the iron-storage protein ferritin and hemosiderin. A proportion of erythrocytes are phagocytosed by activated macrophages. Adapted from (Defrere *et al.*, 2008).

coordinates induction of various defensive genes including detoxifying enzymes and antioxidant proteins, which is critical importance for cellular protection and cell survival (Kaspar *et al.*, 2009).

Activation of the Nrf2-antioxidant responses element (ARE) signaling has been proposed as a major mechanism in the cellular defence oxidative stress and Nrf2 binding to the ARE leads to activation of gene expression (Kaspar *et al.*, 2009; Nguyen *et al.*, 2009). Nrf2 activity is regulated in part by the actin-associated Keap1 (Kelch-like ECH-associating protein 1) protein (Nguyen *et al.*, 2009). Under basal conditions, Nrf2 exists as a complex with the chaperon Keap1 also known as cytosolic inhibitor Nrf2 (INrf2). In response to stimulus, Nrf2 is released from the Keap1/Nrf2 complex and accumulates into the nucleus leading to activation of ARE-mediated transcription of genes such as HO-1 and glutathione S-transferase.

7.1.2 Superoxide Dismutase

Superoxide dismutase (SOD), the first line of defenses against oxygen-derived free radicals, catalyses the dismutation of superoxide into H_2O_2 (Ota *et al.*, 1999b) as shown in *Figure 7.5*. There are three major families of superoxide dismutase, depending on the metal cofactor: Cu/Zn, which binds both copper and zinc, Fe and Mn types, which bind either iron or manganese and finally the Ni type, which binds nickel (Sugino *et al.*, 1996). In human, three forms of superoxide dismutase are available; Cu-Zn SOD (SOD1 that is mainly in cytosol), Mn SOD (SOD2 is located in mitochondria) and extracellular SOD (SOD3 is localized in extracellular fluid) (Rohrdanz *et al.*, 2002). This enzyme is not normally inducible to a great extent. However, it has been found to be overexpressed in the eutopic endometrial of patients with endometriosis (Ota *et al.*, 1999a).

7.1.3 Other antioxidant enzymes

Catalase and glutathione peroxidase (GPx) provide a second line of defence by dismutating peroxide into water and oxygen molecule without the production of free radicals (Wang *et al.*, 2010). In this process, GSH is converted to oxidised glutathione GSSG. In addition, glutathione reductase plays an essential role in maintaining the levels of GSH. This enzyme is ubiquitous and present in all mammalian cells. Glutathione reductase together with its co-factor nicotinamide adenine dinucleotide phosphate (NADPH), catalyses the reduction of GSSG to GSH (Ulusu & Tandogan, 2007). The involvement of these enzymes in defence mechanism is shown in *Figure 7.5*.

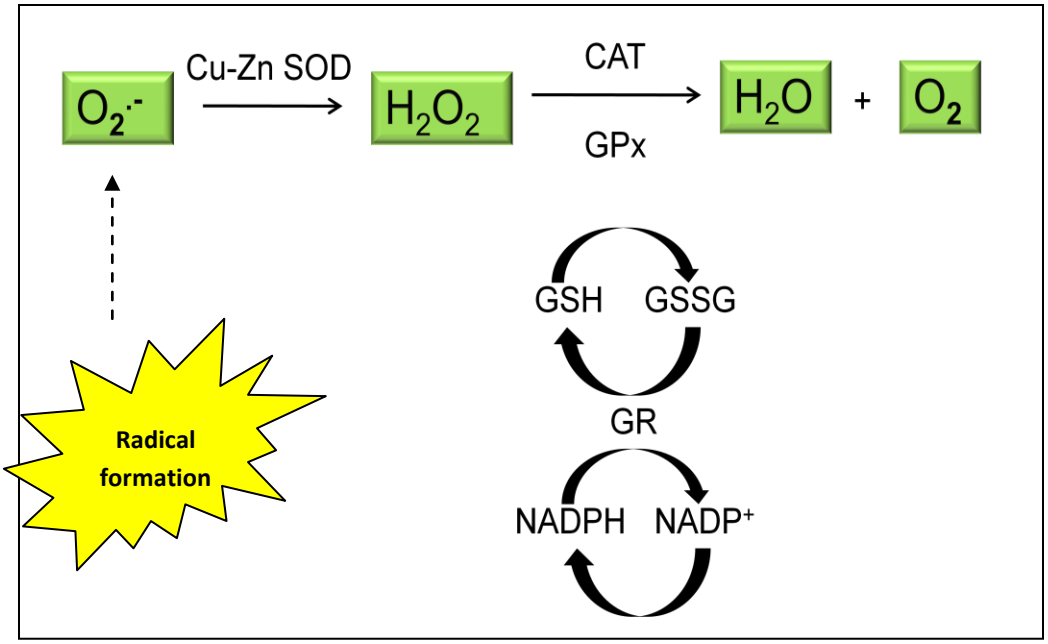


Figure 7.5: Defence mechanism of endogenous antioxidant enzymes. Superoxide dismutase (SOD) catalyses the dismutation of superoxide into H_2O_2. Catalase (CAT) and glutathione peroxidase (GPx) catalyse H_2O_2 to water and oxygen molecule. Glutathione peroxidase GPx also converts GSH to GSSG. GSSG is reduced back to GSH by glutathione reductase (GR). Glutathione reductase utilises NADPH to reduce oxidised glutathione. Adapted from (Willcox *et al.*, 2004).

7.1.4 Chapter Aims

The objective of the work described in this chapter was to determine factors that induce enzymatic antioxidant expression in endometriotic cells and to identify potential biomarker/s in the pathogenesis of endometriosis. Emerging evidence indicates that elevated ROS contributes to the pathogenesis of endometriosis, but the roles of endogenous antioxidant enzymes in the pathogenesis of endometriosis is poorly defined. With evidence implicating the involvement of endogenous antioxidant enzymes in cytoprotection, it is essential to identify the roles of these enzymes in endometriosis, which may lead to valuable therapeutic approaches for limiting the disease progression. In this chapter, only HO-1 and SOD-1 enzymes are studied.

To achieve this aim, this study has set the following objectives:

1. To determine whether oxidants, acrolein or E2 can regulate the expression of the antioxidant enzymes HO-1 and SOD-1
2. To determine HO-1 expression in human samples; ovarian endometriosis and *Uterine myomatosus* as a candidate biomarker
3. To evaluate whether cells develop antioxidant defence mechanism when treated with oxidants, acrolein or E2

7.2 Materials and methods

12-z cells were cultured with oxidants, acrolein, E2 or combination of both E2 with oxidants or acrolein. The concentrations that gave a significant increase in 12-z cell proliferation were used in this chapter. Cells were then analysed for HO-1 and SOD-1 expression. In addition to this chapter, samples from ovarian endometriosis and *Uterine myomatosus* were also used.

All other materials and methods for this chapter are described in chapter 2.

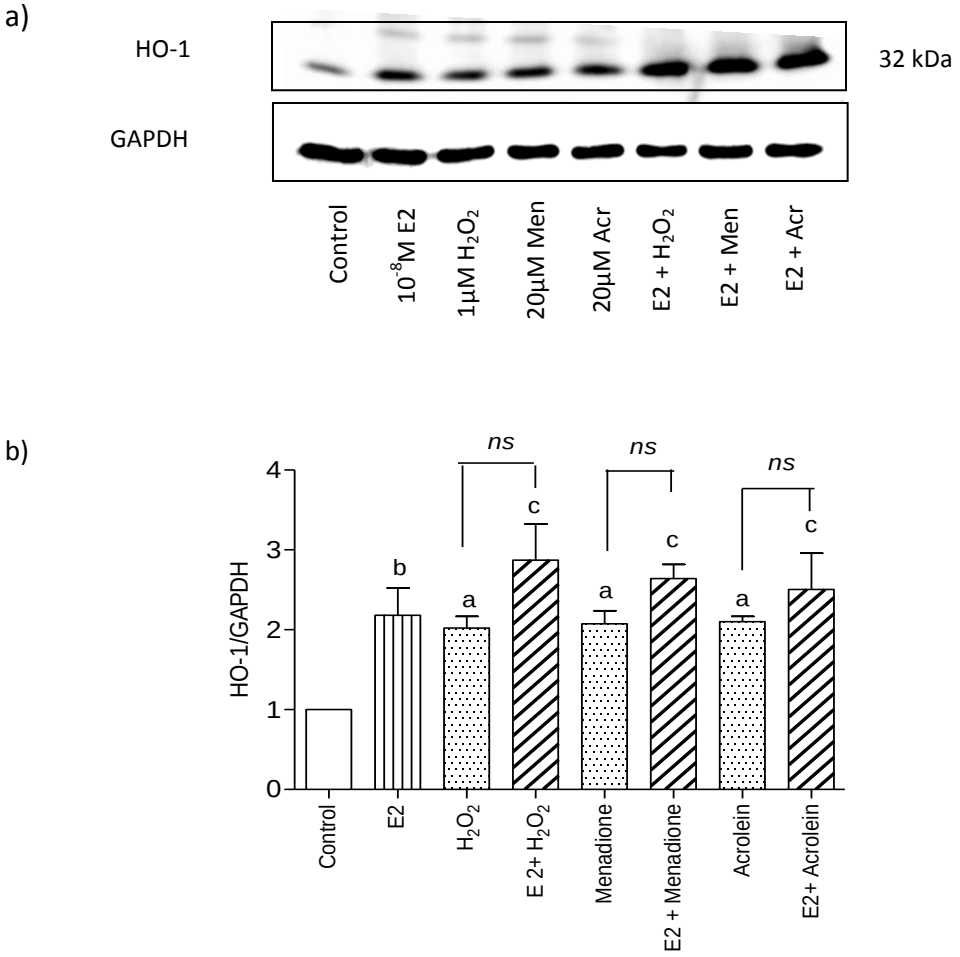
7.3 Results

In order to discover whether antioxidants enhance endometriotic cell survival and to determine whether antioxidant enzymes can be used as a potential biomarker in the pathogenesis of endometriosis, cells were treated with oxidants, acrolein or E2. The expression of antioxidant enzymes, HO-1 and SOD-1 in endometriotic cells were examined. In addition, the expression of HO-1 in samples from ovarian endometriosis and without endometriosis was also examined.

7.3.1 Effects of oxidants, acrolein or E2 on the expression of HO-1 protein

The induction of HO-1 was examined by Western blotting. Cells were treated with 1 µM H_2O_2, 20 µM menadione or 20 µM acrolein for 48 h, and 10^{-8} M E2 for 24 h. Cells were also pre-treated with 10^{-8} M E2 for 24 h followed by addition of oxidants or acrolein at similar concentrations for 48 h. Compared to control, the results show a significant increase in HO-1 expression in all treated cells; E2 (2.2-fold), H_2O_2 (2.0-fold), menadione (2.1-fold) and acrolein (2.1-fold). When cells were pre-treated with E2, the expression of HO-1 was further elevated with the fold increases being; H_2O_2 (2.9-fold), menadione (2.6-fold) and acrolein (2.5-fold). However, there was no significant difference in HO-1 protein expression between cells that were treated with oxidants or acrolein alone as compared to cells that were pre-treated with E2 followed by addition of oxidants or acrolein, as shown in *Figure 7.6*. This indicates that HO-1 is responding to oxidants and acrolein, but E2 has a reinforcing effect on induction. This is the first time that the effect of E2 on HO-1 expression has been investigated in endometriosis.

Figure 7.6: Expression of HO-1 protein in 12-z cells treated with oxidants, acrolein or E2



Expression of HO-1 normalised with GAPDH in 12-z cells treated with H_2O_2 (1 µM), menadione (20 µM) or acrolein (20 µM) for 48 h and in the presence of E2 at 10^{-8} M alone for 24 h. Cells were also pre-treated with 10^{-8} M E2 for 24 h followed by addition of oxidants or acrolein at above concentrations for 48 h. a) Levels of HO-1 enzymes were analysed by Western blotting using HO-1 antibodies and GAPDH as a loading control. b) The protein bands were quantified by image J and expressed relative to GAPDH. Values represent mean ± SEM (n=3).
[a] $p<0.05$, [b] $p<0.01$, [c] $p<0.001$ vs. Control.
ns: not significant

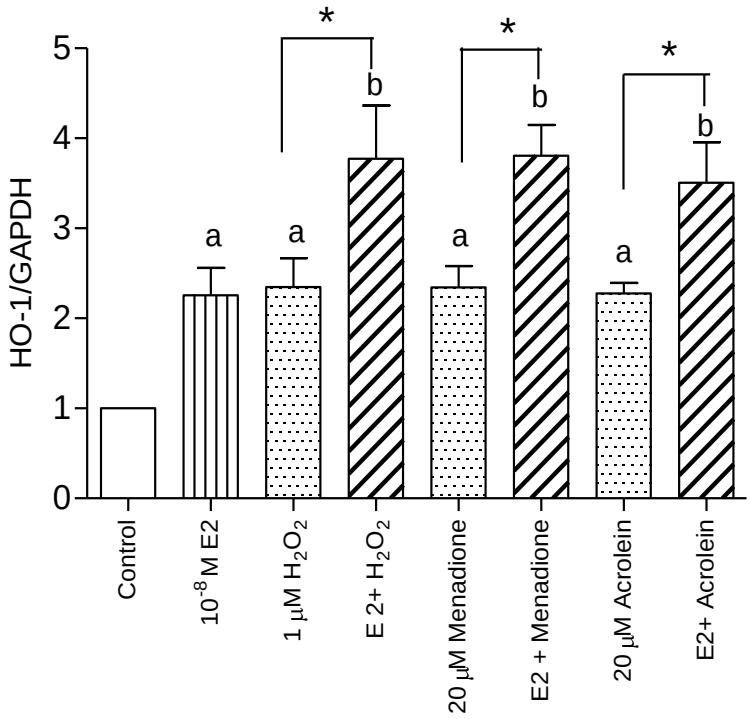
7.3.2 Effects of oxidants, acrolein or E2 on the expression of HO-1 mRNA

To determine whether the observed changes in protein levels were due to changes in mRNA, cells were treated as above and HO-1 mRNA was quantified by Q-RT-PCR using HO-1 oligonucleotides. *Figure 7.7* shows a significant increase in HO-1 mRNA levels in all treated cells with the fold increases were 2.25, 2.30, 2.34 and 2.80 in cells that were treated with E2, H_2O_2, menadione or acrolein respectively when compared to control. In addition, the levels of HO-1 mRNA were much higher when cells were pre-treated with E2 before the addition of H_2O_2, menadione or acrolein with the fold increases were 3.78, 3.8 and 3.5 respectively. These results are consistent with the Western blot findings where HO-1 was upregulated especially in the presence of both E2 and oxidants or acrolein.

7.3.3 HO-1 expression in human samples

To examine whether HO-1 is overexpressed in human endometriotic tissue, levels of HO-1 mRNA were determined by q-RT-PCR in samples from patients with ovarian endometriosis and *Uterine myomatosus* or fibroids as controls. *Figure 7.8* shows a significant increase in HO-1 expression in ovarian endometriosis as compared to controls. The increased levels of HO-1 in endometriotic cells indicates that cells are stressed and this enzyme may play an important role as defence mechanism especially against ROS. Together with the expression results of 12-z cell, HO-1 is proposed as a biomarker in the pathogenesis of endometriosis. However, similar to the results of levels of aromatase in human samples (section 4.3.5), the sample size was too small to infer the levels of this enzyme in the population. Thus, a large number of samples is required in future in order to make a valid inference about the HO-1 levels in patients with and without endometriosis.

Figure 7.7: Expression of HO-1 mRNA in 12-z cells treated with oxidants, acrolein or E2

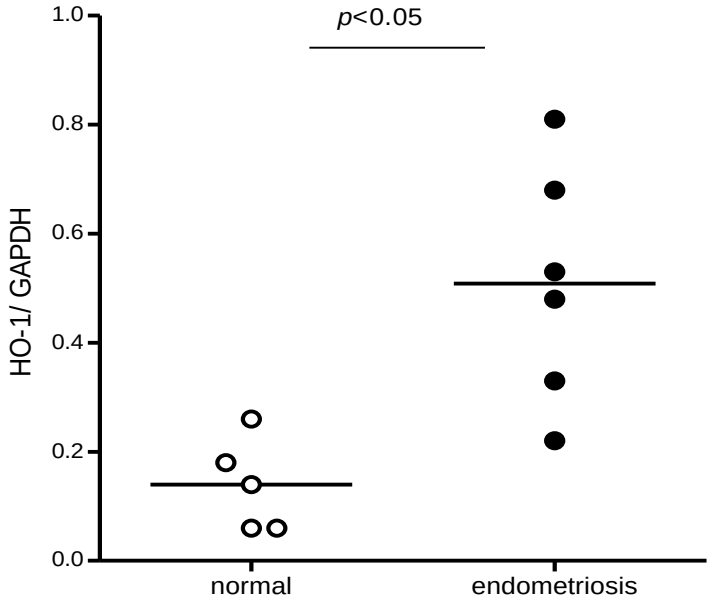


Expression of HO-1 normalised with GAPDH in 12-z cells treated with H_2O_2 (1 μM), menadione (20 μM) or acrolein (20 μM) for 48 h and in the presence of E2 at 10^{-8} M alone for 24 h. Cells were also pre-treated with 10^{-8} M E2 for 24 h followed by addition of oxidants or acrolein at above concentrations for 48 h. Levels of mRNA were quantified by normalisation to the GAPDH as an internal control. Values represent mean ± SEM (n=3).

[a] $p<0.05$, [b] $p<0.001$ vs. Control

* $p<0.05$ oxidants or acrolein alone vs. oxidants or acrolein with E2

Figure 7.8: Expression of HO-1 mRNA in human samples



Expression of HO-1 mRNA in endometriotic cells and controls. The fold changes of mRNA levels were quantified by normalisation to the GAPDH as an internal control, which detected by q-RT-PCR. Values represent mean (n=5; control and n=6; ovarian endometriosis).

7.3.4 Effects of oxidants, acrolein or E2 on the expression of SOD-1 protein

The induction of SOD-1 was examined by Western blotting. Cells were treated with 1 µM H_2O_2, 20 µM menadione or 20 µM acrolein for 48 h, and 10^{-8} M E2 for 24 h. Cells were also pre-treated with 10^{-8} M E2 for 24 h followed by addition of oxidants or acrolein at similar concentrations for 48 h. In contrast to HO-1, there was no significant different in the levels of SOD-1 expression in all treated cells as compared to control, as shown in *Figure 7.9*. This suggests that SOD plays a constitutive role when dealing with oxidants or acrolein.

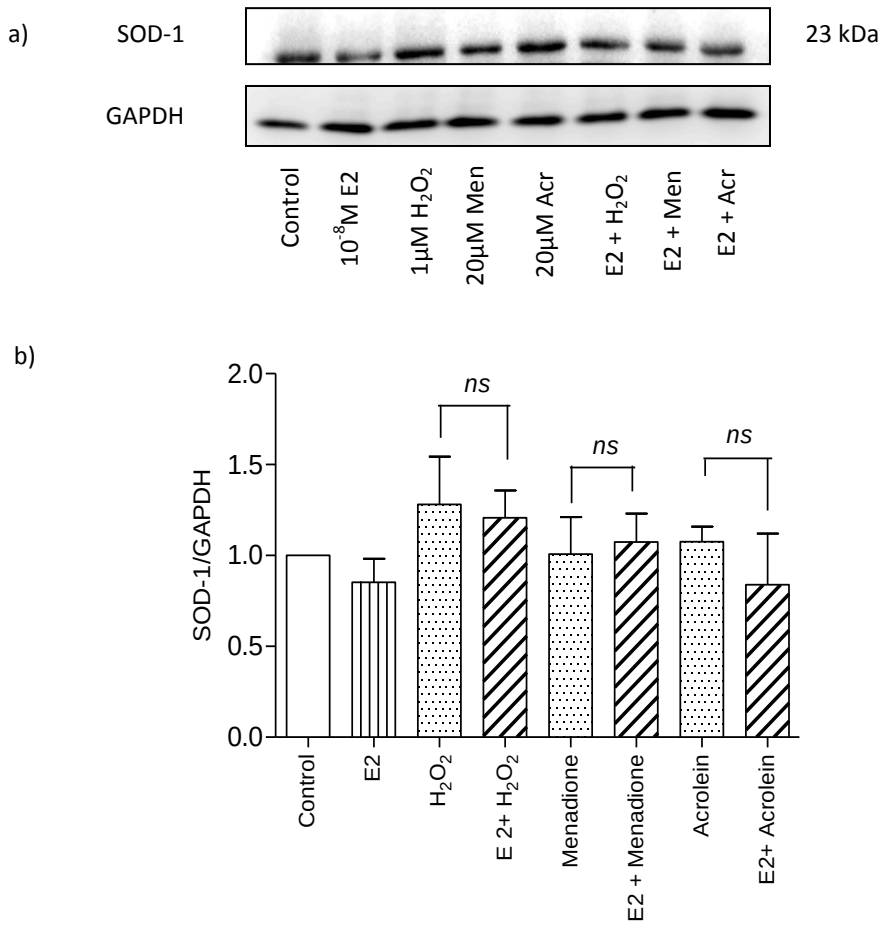
7.3.5 Effects of oxidants, acrolein or E2 on the expression of SOD-1 mRNA

Cells were treated as above and SOD-1 mRNA was quantified by q-RT-PCR using SOD-1 oligonucleotides. There was no significant difference in the levels of SOD-1 expression in all treated cells as compared to control, as shown in *Figure 7.10*.

7.3.6 Effects of oxidants, acrolein or E2 on SOD activity

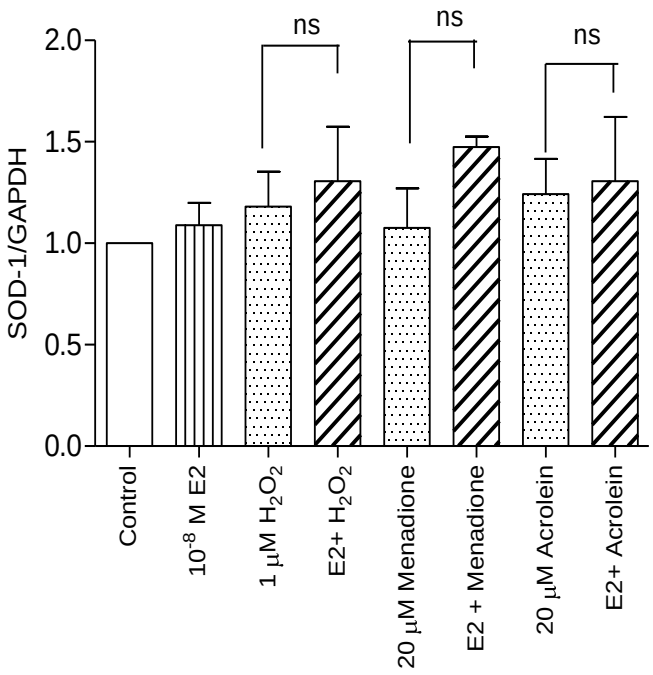
To determine whether SOD activity is influenced by oxidants, acrolein or E2, SOD assay was carried out. *Figure 7.11* shows there was no siginficant difference in the SOD activity in all treated cells as compared to control. The expression results and SOD activity suggest this enzyme is not to be inducible to any great extent by the compounds used and probably less important in the pathogenesis of endometriosis.

7.9 Expression of SOD-1 protein in 12-z cells treated with oxidants, acrolein, E2 and combination of E2 and oxidants or acrolein



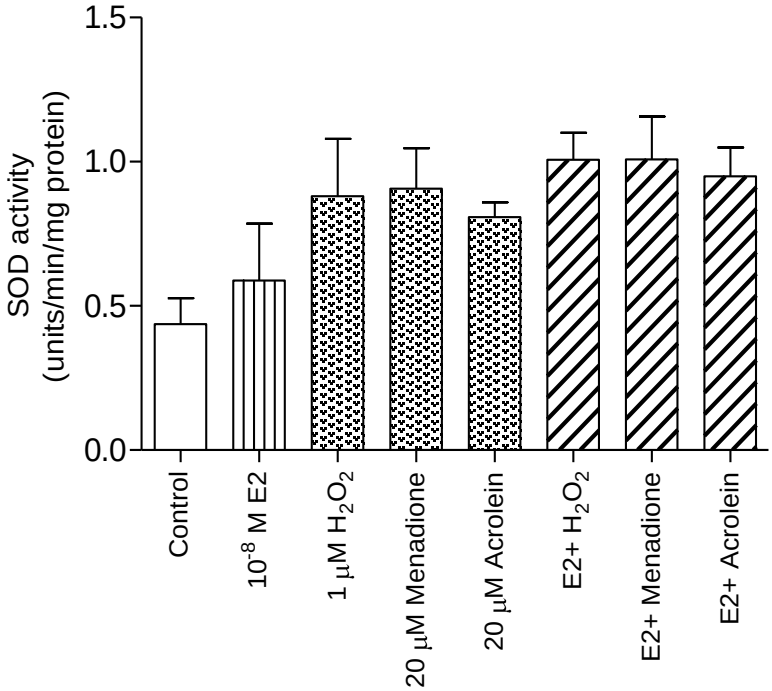
Expression of SOD-1 normalised with GAPDH in 12-z cells treated with H_2O_2 (1 µM), menadione (20 µM) or acrolein (20 µM) for 48 h and in the presence of E2 at 10^{-8} M alone for 24 h. Cells were also pre-treated with 10^{-8} M E2 for 24 h followed by addition of oxidants or acrolein at above concentrations for 48 h. Levels of SOD-1 enzymes were analysed by immunoblotting using SOD-1 antibodies and GAPDH as a loading control. The protein bands were quantified by image J and expressed relative to GAPDH. Values represent mean $\pm$ SEM (n=3).
ns: not significant

Figure 7.10: Expression of SOD-1 mRNA in 12-z cells treated with oxidants, acrolein, E2 and combination of E2 and oxidants or acrolein



Expression of HO-1 normalised with GAPDH in 12-z cells treated with H_2O_2 (1 μM), menadione (20 μM) or acrolein (20 μM) for 48 h and in the presence of E2 at 10^{-8} M alone for 24 h. Cells were also pre-treated with 10^{-8} M E2 for 24 h followed by addition of oxidants or acrolein at above concentrations for 48 h. Levels of mRNA were quantified by normalisation to the GAPDH as an internal control. Values represent mean ± SEM (n=3).
ns: not significant

Figure 7.11: Effects of oxidants, acrolein, E2 and combination of E2 and oxidants or acrolein on SOD activity in 12-z cells



SOD activities in 12-z cells treated with H_2O_2 (1 μM), menadione (20 μM) or acrolein (20 μM) for 48 h and in the presence of E2 at 10^{-8} M alone for 24 h. Cells were also pre-treated with 10^{-8} M E2 for 24 h followed by addition of oxidants or acrolein at above concentrations for 48 h. Values represent mean ± SEM (n=3).

7.3.7 Adaptive response of endometriotic cells to oxidants and acrolein

To evaluate whether cells developed antioxidant protection against oxidants or acrolein following pre-treatment of these compounds and induction of HO-1, cells were plated in 96-well plates for 24 h cells and pre-treated with these compounds at the concentrations that caused induction of HO-1 enzymes. In addition, cells were also treated with E2 to determine whether antioxidant has any effects when cells were exposed to E2. A killing curve was developed following treatment. Cell viability was measured by MTT assay and the results were compared with non pre-treated cells (without any treatment).

7.3.7.1 Antioxidant protection against H_2O_2

To test whether pre-treatment with H_2O_2 or E2 leads to a protective response, cells were treated with 1 µM H_2O_2 for 48 h or 10^{-8} M E2 for 24 h and/or pre-treated with E2 for 24 h followed by addition of 1 µM H_2O_2 for 48 h. Subsequently, cells were exposed to H_2O_2 (0.5 µM-500 µM) and cultured for 24 h. As shown in *Figure 7.12(a)*, in cells that were not pre-treated, the percentage of cell viability decreased when H_2O_2 concentrations increased. H_2O_2 pre-treated cells showed a greater survival than non pre-treated cells when cells were exposed to 10 µM-50 µM H_2O_2 at the end of the pre-treatment. Thus, it is proposed that cells developed antioxidants defence mechanisms when exposed to H_2O_2, which protected them against subsequent H_2O_2 toxicity. This is evidence of an adaptive response to H_2O_2. However, E2 did not protect the cells even though it increases cell proliferation.

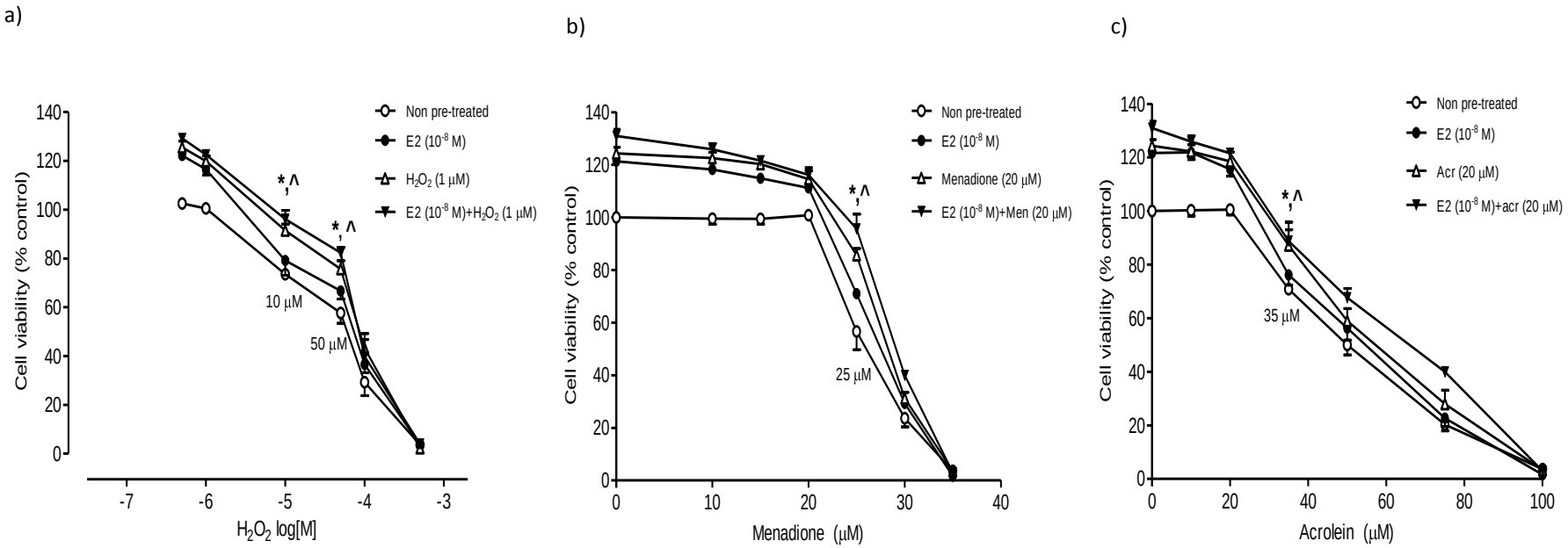
7.3.7.2 Antioxidant protection against menadione

Cells were pre-treated with 20 µM menadione for 48 h or 10^{-8} M E2 for 24 h and/or pre-treated with E2 for 24 h followed by addition of 20 µM menadione for 48 h. Subsequently, menadione (10 µM-35 µM) was added to each respective well and cultured for 24 h. As shown in *Figure 7.12(b)*, in cells that were not pre-treated, the percentage of cell viability decreased when menadione concentrations increased. Menadione pre-treated cells showed a greater survival than non pre-treated cells when cells were exposed to 25 µM menadione at the end of the pre-treatment. Thus, it is proposed that cells developed antioxidants defence mechanisms when exposed to menadione, which protected them against subsequent menadione toxicity. This is evidence of an adaptive response to menadione. Similarly, E2 did not protect the cells even though it increases cell proliferation.

7.3.7.3 Antioxidant protection against acrolein

In this experiment, cells were pre-treated with 20 µM acrolein for 48 h or 10^{-8} M E2 for 24 h and/or pre-treated with E2 for 24 h followed by addition of 20 µM acrolein for 48 h. Subsequently, cells were exposed to acrolein (10 µM-100 µM) and cultured for 24 h. In cells that were not pre-treated, the percentage of cell viability decreased when acrolein concentrations increased. As shown in *Figure 7.12(c),* acrolein pre-treated cells showed a significant increased in cell viability than non pre-treated cells when cells were exposed to 35 µM acrolein at the end of the pre-treatment. Thus, it is proposed that cells developed antioxidants defence mechanisms when exposed to acrolein, which protected them against acrolein toxicity. This is evidence of an adaptive response to acrolein, but E2 did not show any protective effect against acrolein.

Figure 7.12: Killing curve plots for 12-z cells in response to oxidants, acrolein, E2 and combination of E2 and oxidants or acrolein



Cells were exposed to 1 μM H_2O_2, 20 μM menadione or 20 μM acrolein for 48 h, 10 $^{-8}$ M E2 for 24 h or pre-treatment with 10 $^{-8}$ M E2 for 24 h followed by addition of oxidants or acrolein for 48 h. Killing curve was determined by exposing the cells with a) H_2O_2 b) menadione and c) acrolein at concentrations shown for 24 h. Cell viability was measured using the MTT assay. Values represent mean ± SEM (n=3).

* p<0.05 oxidant or acrolein alone vs. non pre-treated

^ p<0.05 E2 and oxidant or acrolein vs. non pre-treated

7.4 Discussion

This chapter aimed to determine whether HO-1 and SOD1 enzymes are inducible by oxidants, acrolein or E2, and to identify whether these enzymes are good biomarkers for endometriosis. A biomarker is a substance that can provides a great opportunity to recognize a disease, pharmacological responses to a therapeutic intervention as well as provide information for assessing clinical outcomes of treatment. In addition, this study also aimed to test whether induction of an adaptive response leads to significant protection against oxidants and acrolein. Although both of these enzymes have been implicated in the pathogenesis of endometriosis, there is little published data available especially on HO-1 in endometriosis.

7.4.1 HO-1 is inducible in endometriotic cells

Considerable research efforts have been devoted to understand the significant role of HO in cell protection against elevated ROS. The inducible isoform of haem oxygenase (HO-1) has been proposed as an effective system to counteract the presence of elevated ROS *in vivo*. The present study shows for the first time, that E2, oxidants (H_2O_2 and menadione) and also acrolein can induce HO-1 expression in endometriotic cells. HO-1 expression was shown to be significantly induced by E2, oxidants and acrolein at both the mRNA level and the protein level especially the combination of both E2 and oxidants or acrolein. The role of estrogen in HO-1 induction in human has not been well clarified, but it has been demonstrated that estrogen was ineffective in inducing HO activity in rat testis (Sadler *et al.*, 1986).

The HO-1 gene is commonly induced by agents and chemicals that produce an oxidative cellular stress (Ryter *et al.*, 2006) supporting the observed upregulation of HO-1 in this study. A diverse array of stimuli that mainly induce elevated ROS has

been shown to induce HO-1 expression. Therefore, the expression of this enzyme *in vivo* represents an endogenous antioxidant as a protective response. Although HO-1 has been reported to protect cells from elevated ROS by its enzymatic activity, the functions of this enzyme in endometriosis has not been completely elucidated. However, the inducibility suggests that endometriotic cells are capable of adapting to withstand oxidative insult and may explain why these cells survive and proliferate under oxidative conditions.

Although many diseases have been associated with increased expression of HO-1 such as Alzheimer's disease (Schipper, 2011), diabetes mellitus and atherosclerosis (Abraham & Kappas, 2005), there is only one known study that has looked at the expression of HO-1 in endometriotic cells (Van Langendonckt *et al.*, 2002a). In that study, RT-PCR analysis of biopsies of endometriotic cells and eutopic endometrium from patients with endometriosis was done. This demonstrated that HO-1 was strongly expressed in endometriotic cells, shown by band intensity on agarose gel. In addition, HO-1 expression normalised to β-actin expression appeared to be higher in endometriotic cells than in eutopic endometrium, similar to our results although our controls were untreated endometriotic cells. Van Langendonckt and colleagues also measured free haemoglobin and the product of haemoglobin breakdown, total and direct bilirubin in peritoneal fluid and serum of women with and without endometriosis. A significant increase in free hemoglobin concentrations, but not total or direct bilirubin was detected in peritoneal fluid and serum of women with endometriosis as compared to women without endometriosis. However, the reason of no significant different in the levels of bilirubin was not clarified, which raises the question as to how high HO-1 levels are protecting against ROS.

7.4.2 HO-1 as a potential biomarker in endometriosis

The up-regulation of HO-1 protein and mRNA indicates that this enzyme can be used as a reliable marker in the pathogenesis of endometriosis. Its expression in endometriotic cells indicates that these cells are likely to be experiencing stress conditions, leading to the induction of HO-1. As endometriosis causes a cyclic hemorrhage in the peritoneal cavity, this enzyme is proposed to have a pivotal role to detoxify haem. There is lack of data on how the peritoneal environment copes with the presence of red blood cells and their deleterious byproducts. Expression of HO-1 is clearly one way in which these cells can adapt.

7.4.3 Low induction of SOD-1 in endometriosis

Unlike HO-1 enzyme, there was no significant difference in the protein level or the mRNA level of SOD-1 in all treated 12-z cells as compared to control. In addition, there was no significant different in the SOD activity. There is no previous study that has looked at the direct effects of E2, oxidants and acrolein on the expression and activity of SOD. The SOD expression and activity results indicate that although SOD is present, it is not inducible by E2, oxidants or acrolein. It is therefore unlikely that it is inducible in endometriotic cells and is unlikely to represent a useful biomarker for endometriosis. This is in contrast to an earlier study where SOD expression was examined using immunohistochemical assessment comparing patients with endometriosis and fertile women as controls where endometrial biopsies at different phases of menstrual cycle was obtained (Ota *et al.*, 1999b). These authors determined a significant increase in SOD expression throughout the menstrual cycle in the endometrium of patients with endometriosis as compared to controls without significant changes among the phases. Thus, SOD appeared persistently high in endometriotic cells. However, there is no mechanistic explanation to this finding. The role of SOD in endometriosis was further supported

by several lines of evidence indicating that levels of cytokines including interleukin (IL)-1 and tumor necrosis factor (TNF) are increased in the peritoneal fluid of women with endometriosis, and these cytokines are potent inducers of SOD expression (Ota *et al.*, 1999b). However, significantly low levels of SOD was found in peritoneal fluid of women with endometriosis as compared to normal women, the reason for which is not clear (Szczepanska *et al*., 2003).

7.4.4 HO-1 as one of the cytoprotective enzymes against ROS and acrolein

To combat the deleterious effects of ROS and allow oxidative cellular metabolism, aerobic organisms have developed protective antioxidant enzymes. This chapter also demonstrated 12-z cells developed an adaptive response to oxidants and acrolein when exposed to low levels of those compounds. The increased viability comparing to non pre-treated cells was observed when cells were exposed to moderate concentrations of oxidants or acrolein, as seen in the killing curve. It is apparent that antioxidant protection may not develop when cells were exposed to E2 unless in combination with oxidants or acrolein. However, the antioxidant protection may involved various other endogenous antioxidant enzymes, with HO-1 is proposed as one the cytoprotective enzymes as its expression was upregulated by the compounds used in this study.

In summary, this chapter has identified HO-1 as a potential biomarker in the pathogenesis of endometriosis. Its expression was induced by E2, oxidants and acrolein. HO-1 was significantly upregulated in samples of ovarian endometriosis. Although SOD1 is considered as one of the main constitutive protective enzymes, its expression was not found to be inducible to any great extent.

CHAPTER EIGHT:

GENERAL DISCUSSION

AND

CONCLUSION

8.1 General discussion

Endometriosis is a chronic disease with the major symptoms are pain and infertility. Research into the underlying molecular and cellular mechanisms of endometriosis has been conducted over many years. However, the pathogenesis of this disease remains an enigma. The theory of *'retrograde menstruation'* by John Sampson in 1921 represents a plausible explanation for the development of endometriosis, which describes that desquamated endometrial cells are transported into the peritoneal cavity, and the still-viable cells subsequently implant, grow and proliferate (Vinatier *et al.*, 2001; Flores *et al.*, 2007). However, factors that contribute to endometriotic cell growth and proliferation remain elusive. Immune and inflammatory alterations at the cellular and molecular levels in endometriosis have been implicated for the endometriotic survival and growth (Flores *et al.*, 2007). As an estrogen dependent disease, estrogen plays a crucial role in the mechanism of cell growth (Zeitoun & Bulun, 1999; Izawa *et al.*, 2008). In addition, endometriosis is associated with ROS that modulate endometriotic cell proliferation (Shanti *et al.*, 1999; Agarwal *et al.*, 2003; Ngo *et al.*, 2009). This disease also remains a major therapeutic challenge, mainly because the aetiology is not well understood.

Although surgery or medical treatment can improve endometriosis, the disease tends to recur. Furthermore, most of the current medical treatment for endometriosis, including non-steroidal anti-inflammatory agents, combined oral contraceptives, androgenic agents and gonadotropin-releasing hormone analogues, have significant side effects. To establish the mechanism of endometriotic cell proliferation, human immortalized endometriotic epithelial cells, 12-z were used in this study. Factors that induced and inhibited cell growth were also identified. It is interesting to note that this study was able to determine changes in gene expression in 12-z cells that are induced by oxidative stress, its byproducts and E2.

Cell signaling pathway/s that are involved in the mechanism of proliferation and inhibition of endometriotic cell growth were also investigated.

8.1.1 Oxidants, acrolein and E2 induced cell proliferation in 12-z cells

Numerous factors have been implicated in endometriotic cell growth. The results in Chapter 3 revealed a significant increased in cell proliferation in the presence of low concentrations of oxidants and lipid peroxidation products revealing a possible role for oxidants and acrolein in the regulation of endometriotic cell growth. Conversely, at high concentrations, oxidants and acrolein inhibited cell growth. Thus, similar to previous studies that investigated H_2O_2 only (Foyouzi *et al.*, 2004), we have demonstrated a biphasic dose response with not only H_2O_2, but also menadione and acrolein. This is clinically relevant because low levels of ROS and aldehyde lipid peroxidation products such as malondialdehyde are known to occur in the peritoneal fluid of women with endometriosis or are released by the endometriotic lesions, and represent factors that induce endometriotic cell proliferation.

A significant increase of oxidative stress markers in peritoneal fluid (Murphy *et al.*, 1998) and systemic circulation (Shanti *et al.*, 1999; Lambrinoudaki *et al.*, 2009) have been demonstrated earlier in women with endometriosis as compared to normal women, which led to the proposal of an association between oxidative stress and endometriosis. However, some investigators did not find any association (Ho *et al.*, 1997; Bedaiwy *et al.*, 2002). In future work, levels of superoxide anion ($O_2^{\cdot-}$) and H_2O_2 should be considered for measuring in biological fluids (serum and peritoneal fluid) to further confirm whether these factors are involved in the mechanism of cell proliferation in endometriosis. However, some authors have reported the difficulty of measuring ROS *in vivo* as they are highly reactive and relatively unstable

(Thannickal & Fanburg, 2000). Previously, both of these ROS have been demonstrated to be significantly higher in endometriotic cells as compared to endometrium (Ngo *et al.*, 2009). Elevated levels of aldehydes including acrolein are also considered a potent marker for enhanced oxidative stress and the intracellular acrolein concentrations have been measured in human breast cancer cells (Kato *et al.*, 2002). However, acrolein has never been measured in endometriosis. Determination of ROS and acrolein levels in women with endometriosis would provide further insight into the relationship between endometriosis and oxidative stress.

In addition to oxidants and acrolein, the results in Chapter 4 showed that 10^{-8} M E2 significantly induced cell proliferation in 12-z cells. The additive effects of E2 and oxidants or acrolein were found to further enhance cell proliferation, which again is clinically relevant as E2 and elevated ROS are likely to co-exist in endometriosis. The absence of cell proliferation at physiological concentration of E2 (10^{-9} M) support the facts that increased levels of E2 is required for endometriotic cells to proliferate that mimics the physiological situation in endometriosis. Thus, measuring levels of serum E2 in women with and without endometriosis is proposed in order to further support the roles of E2 in endometriosis.

8.1.2 A *'positive feedback loop'* for E2 biosynthesis in 12-z cells

Endometriosis is a disease associated with aberrant gene expression. To further determine the roles of above factors in the mechanism of endometriotic cell proliferation, the expression of estrogen biosynthesis and metabolising enzymes and factor/s involved in their productions were measured in response to oxidants, acrolein and E2 at concentrations that produced significant effects in cell proliferation. These genes included aromatase, COX-2, 17β-HSD type 1, 17β-HSD

type 2, AKR1C3 and factors that are involved in estrogen production, and the level of PGE_2 was also measured. Generally, the expression of aromatase, 17β-HSD Type 1, AKR1C3 and COX-2 was seen to be upregulated by E2, oxidants and acrolein, especially the combination of both E2 and oxidants or acrolein, which was determined by Western blotting and q-RT-PCR. In our model, the mechanism of E2 production (the main form of estrogen) is described as follows: E2 stimulates the action of COX-2, which then increases the PGE_2 synthesis. In turn, an increase in PGE_2 production activates aromatase activity that produces high amounts of estrone. In the presence of high levels of 17β-HSD type 1 induced by E2, estrone is converted to the more active E2. This finding of a *'positive feedback loop'* for E2 biosynthesis in 12-z cells that induced by E2, oxidants and acrolein provides a constant source of E2 to the cells, which has been shown in *Figure 4.16*. Conversely, the expression of 17β-HSD Type 2, which is known to convert E2 back to estrone was found to be not significantly altered in all treated cells as compared to control. This may explain the increased levels of E2 that promotes cell growth. Indeed, each of the enzymes has its own potential role in E2 biosynthesis or inactivation. Moreover, this indicates the estrogen biosynthesis enzymes are of paramount importance in the mechanism of endometriotic cell proliferation. Previously, a positive feedback loop was discovered within endometriotic stromal cells (Zeitoun *et al.*, 1998) and our work supports this observation in endometriotic epithelial cells.

Aromatase, a key enzyme in estrogen synthesis appears to play an important role in endometriosis (Zeitoun & Bulun, 1999; Attar & Bulun, 2006a; Banu *et al.*, 2008). The successful treatment of endometriosis using an aromatase inhibitor (Ailawadi *et al.*, 2004; Razzi *et al.*, 2004) obviously support the involvement of aromatase in the pathogenesis of endometriosis. We showed a clear increase in aromatase activity as well as expression. A significant upregulation of aromatase expression in endometriotic samples from women with ovarian endometriosis as compared to

endometrium from women with *Uterine myomatosus* further support the idea that this enzyme plays an important role in E2 biosynthesis, thus this study provides an evidence that aromatase inhibitor has a potential role in the treatment of endometriosis. The expression of other biosynthesis enzymes in human samples from different stages of endometriosis should be considered in future, which will give a better picture of their levels in endometriosis as compared to women without endometriosis.

8.1.3 Mechanism of action of E2 to cause cell proliferation

Conflicting evidence has been presented on which ER subtypes are involved in the mechanism of estrogen action in endometriosis, either ER-α (Matsuzaki *et al.*, 2001; Banu *et al.*, 2009) or ER-β (Brandenberger *et al.*, 1999; Hudelist *et al.*, 2005; Xue *et al.*, 2007). Our study proposed ER-α is involved in the mechanism of cell proliferation and the nuclear accumulation of this receptor was induced by oxidants, acrolein and E2, as demonstrated by Western blotting and q-RT-PCR. Therefore, it is suggested that a significant increase of ER-α in endometriotic epithelial cells induced by these compounds might stimulate the disease progression and increase the rate of proliferation. Similar to most of the genes that are measured in this study, the expression of this receptor was further enhanced in the presence of E2 with oxidants or acrolein. ER-α mediates the E2 action either via ERE-dependent or non ERE-dependent mechanisms as been shown in Figure 5.15. The use of ER-α antagonist in future studies would further confirm the involvement of this receptor. ERs are an important target for the development of drugs to prevent breast cancer, another estrogen-dependent disease, where the interaction of estrogen with the ER can stimulate cell proliferation. For example, tamoxifen and raloxifen (Selective estrogen receptor modulators-SERMs) are competitive inhibitors of E2 at the ERs. Extensive studies are needed to determine whether the same concept used breast cancer treatment can be applied to endometriosis.

8.1.4 MAPK/ERK1/2 and PI3K/Akt are involved in the mechanism of endometriotic cell proliferation

To date, published data on the signaling pathways that are involved in cell proliferation in endometriosis have been inconsistent. To further examine the possible signaling pathway/s that are involved in the mechanism of endometriotic cell proliferation, the activation of MAPK/ERK1/2 and PI3K/Akt were studied. The results of Chapter 5 revealed both of the pathways that are involved in the mechanism of endometriotic cell proliferation, as determined by MTT assays and Western blotting. By using the ERK1/2 inhibitor (PD98059) and PI3K inhibitor (LY294002), this study strikingly demonstrated that E2 activates both the MAPK/ERK1/2 and PI3K/Akt signaling pathways. In endometriosis, E2 actions also involves non ERE-dependent pathways including MAPK/ERK1/2 (Murk *et al.*, 2008; Zhang *et al.*, 2010), p38 MAPK (Seval *et al.*, 2006) and PI3K/Akt (Zhang *et al.*, 2010) signalling pathways. We propose that in addition to the ERE-dependent pathway, the mechanism of E2 induced endometriotic cell proliferation also involves MAPK/ERK1/2 and PI3K/Akt signaling pathways with ER-α as the responsible receptor. The effects of inhibitors on oxidants treated cells indicated the involvement of MAPK/ERK1/2 pathway, but not p13K/Akt. The activation of MAPK/ERK1/2 pathway by H_2O_2 (Yoshino *et al.*, 2004) and cytokines (Grund *et al.*, 2008) has been shown previously in endometriotic cells. Although acrolein has been shown to promote cell proliferation, the signaling pathway/s that are evoked by this compound needs further investigation. Further studies are needed to identify the possible involvement of other kinase/s that may phosphorylate Akt despite PI3K. In addition, the use of other PI3K inhibitor (Wortmannin) may be considered in future as this inhibitor has different properties as compared to LY294002. Other signaling pathways should be considered in future such as NF-κB and p38 MAPK signaling pathways as both have been shown to be involved in the stimulation of endometriotic cell growth (Seval *et al.*, 2006; Zhang *et al.*, 2010).

Research on agents inhibiting the signaling pathways as a potential for endometriosis treatment should be explored in future studies.

8.1.5 NAC has the potential effects to inhibit endometriotic cell proliferation

Having established the ability of oxidants to induce endometriotic cell proliferation, we next investigated whether antioxidant NAC can inhibit this process. The results in Chapter 6 demonstrated that 3 mM-30 mM the antioxidant NAC significantly inhibited 12-z cell growth and at higher concentrations increased LDH leakage suggestive of irreversible cell death or necrosis. Thus, a reduction in cell viability by 3 mM NAC appeared to be due to apoptotic cell death and there was no significant LDH leakage at this concentration. Previous studies demonstrated that 10 mM NAC inhibited cell growth in endometrial stromal cell from women with endometriosis (Foyouzi *et al.*, 2004) and in YHES cell (Wu & Guo, 2006). The fluorescence microscopic findings of an apoptosis study further support NAC induced apoptosis especially at 3 mM. To further elucidate the mechanism of apoptosis by NAC, subcellular fractionation was done and apoptotic biomarkers including Bax, activated caspase 3 and cytochrome c were identified in NAC treated and untreated cells. An increase in expression of these biomarkers in NAC treated cells was observed especially at 3 mM. We determined that the mechanism of NAC-induced apoptosis in 12-z cells involved mitochondrial dependent and JNK signaling pathways.

JNK mediates NAC induced apoptosis through Bax activated mitochondrial dependent pathway. In addition to mitochondrial dependent and JNK signaling pathways, it is possible that the extrinsic pathway, death-ligand receptor pathway may also involved as NAC has been shown to activate the expression of caspase 3 persistently even in the presence of JNK inhibitor, SP600125. NAC induced apoptotic pathway through death-ligand receptor pathway in YHES cell line has

been shown previously (Wu & Guo, 2006). The role of NAC as an antioxidant was supported by the inhibitory effects of H_2O_2 induced proliferation similar to previous studies (Foyouzi *et al.*, 2004; Wu & Guo, 2006) and its ability to improve the redox balance of GSH/GSSG that was disrupted by H_2O_2.

As endometriosis is an estrogen dependent disease, standard medical treatments have been aimed at inducing hypoestrogenism and inducing atrophy of endometriotic implants (Huang, 2008). Thus, medical treatment mostly used in endometriosis includes hormonal suppressive therapy, which downregulates the hypothalamus-pituitary-ovarian (HPO) pathway. Although data reported in the literature have suggested that oxidative stress is responsible in part for the development of progression of endometriosis (Shanti *et al.*, 1999; Agarwal *et al.*, 2003; Ngo *et al.*, 2009) and a decrease in oxidative stress markers was observed in the peripheral blood of women with endometriosis after antioxidants supplementation (Mier-Cabrera *et al.*, 2009), antioxidants have not been included in the standard treatment for this disease. From our findings we would like to propose that NAC can be used as an alternative drug in addition to the current medical treatment. Furthermore, NAC is very stable, easily enters the cell and is rapidly deacetylated into cysteine and subsequently converted into GSH (Sen, 1998). As endometriosis needs a long period of treatment, only medications with a favourable safety, tolerability, efficacy and cost profile should be considered (Vercellini *et al.*, 2011). It has been proposed previously that treatment strategies for endometriosis should be directed toward lowering of ROS levels (Agarwal *et al.*, 2003). In addition, one of the mechanisms by which some drugs act on the clinical control of endometriosis is by a reduction of cell proliferation and an increase of apoptosis (Gomes *et al.*, 2009). Most recently, it has been proposed that apoptosis-inducing agents could be a potential therapeutic strategy for the treatment of endometriosis (Kim *et al.*, 2011b). A summary of the potential drug therapies for endometriosis are shown in *Figure 8.1*.

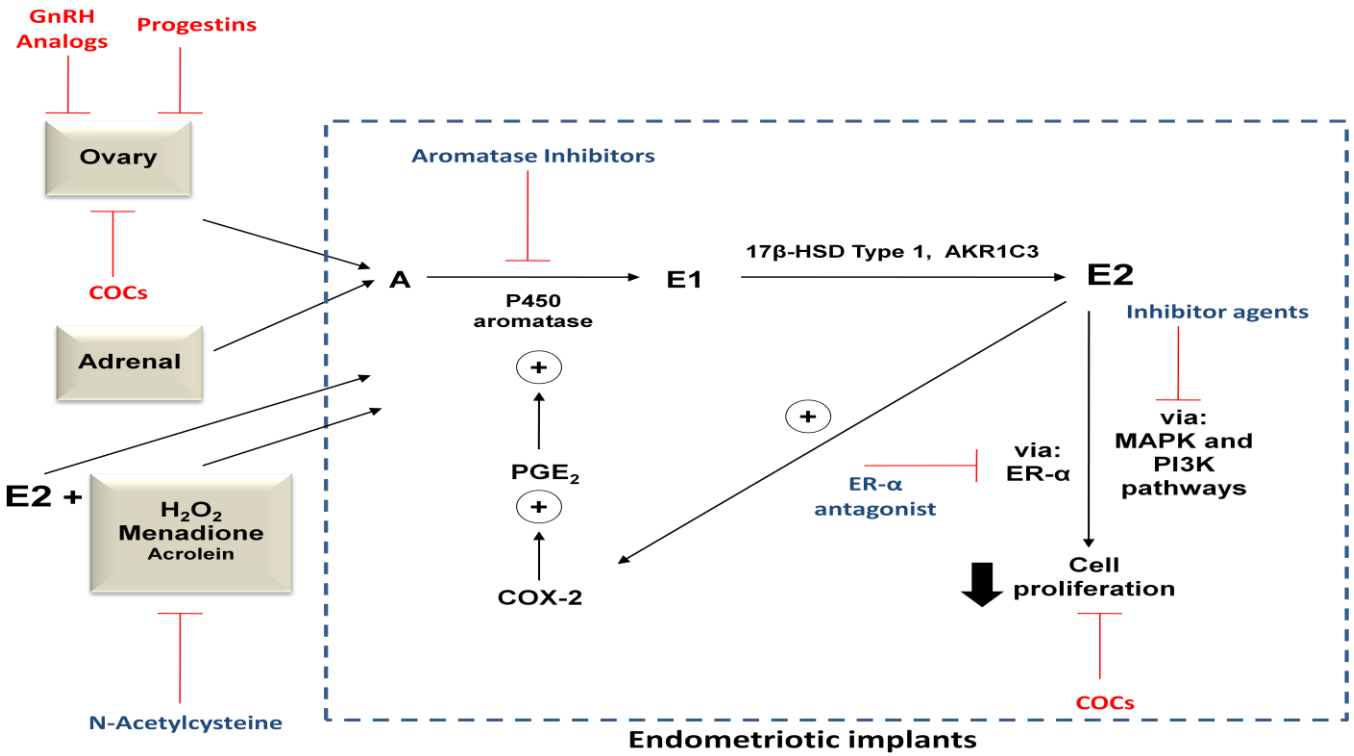


Figure 8.1: The schematic diagram of the mechanism of several drugs for endometriosis. Standard (in red) and experimental (in blue) medical treatments in endometriosis. GnRH analogues and progestins suppress hypothalamic-pituitary-ovarian (HPO) axis that reduce the systemic estrogen levels. Combined oral contraceptive pills (COCs) prevent ovulation and inhibit cell proliferation. Aromatase inhibitors inhibit the conversion of androstenedione to E1, thus decrease the E2. ER-α antagonist antagonise or inhibit the effects of E2 as well as MAPK and PI3K inhibitors. These mechansims result in decrease E2 production and subsequently inhibit cell proliferation.
GnRH: Gonadotrophin Releasing Hormone, COCs: Combined oral contraceptive pills, A:androstenedione, E1: Estrone. E2: Estradiol, COX-2: cyclooxygenase-2, PGE_2: prostaglandin E_2.

8.1.6 HO-1 as a potential biomarker for endometriosis

Organisms have developed a system of defence mechanism including antioxidants, antiapoptosis proteins, detoxifying enzymes and survival signaling pathways to counteract various toxic and environmental stresses (Roy *et al.*, 2009). Oxidative stress may developed as a consequence of excessive production of ROS and/or impaired antioxidant defence mechanisms (Agarwal *et al.*, 2003). The mechanism of protection from ROS including limiting the production of ROS, inactivating ROS, and repairing cell damage. Besides using the exogenous antioxidant NAC, this study also determined roles of the endogenous antioxidants enzymes, HO-1 and SOD1. This study has identified HO-1 as a potential biomarker in the pathogenesis of endometriosis, which was supported by expression studies of 12-z cells. Oxidants, acrolein and E2 significantly induced HO-1 expression especially the combination of E2 and oxidants or acrolein. An apparent increased of HO-1 in samples from women with ovarian endometriosis as compared to controls further support an important role of this enzyme. A biomarker could help in making the diagnosis of endometriosis and also allow the effects of treatment to be monitored. This study was strengthened by findings that induction of enzymes such as HO-1 may be able to protect cells where an adaptive response was observed when cells were exposed to low levels of oxidants and acrolein.

In conclusion, based on our study, endometriotic epithelial cells play an important role in endometriotic cell growth. Elevated ROS modulating cell proliferation that has been demonstrated in 12-z cell line may support the evidence that oxidative stress induce cell proliferation in endometriosis. Hence our data strongly support the concept that endometriosis is closely related to the presence of estrogen and excessive oxidative stress, which have potential relevance to the pathogenesis of endometriosis. We have demonstrated that the mechanism of endometriotic cell proliferation involves various factors including oxidants, acrolein and E2. These

compounds especially the combination of E2 and oxidants or acrolein enhanced the expression of various estrogen biosynthesis and enzymes that form a *'positive feedback loop'* and results in increase of E2 biosynthesis. The mechanism of cell proliferation by these compounds involves MAPK/ERK1/2 and PI3K/Akt signaling pathways. The apoptotic induction by NAC, its inhibitory effects on H_2O_2 induced cell proliferation and its ability to improve the redox balance of GSH/GSSG may contribute to potential therapeutic strategies for endometriosis.

References

Abraham, N.G. & Kappas, A. (2005). Heme oxygenase and the cardiovascular-renal system. Free Radic Biol Med. 39**:** 1-25.

Abraham, N.G. & Kappas, A. (2008). Pharmacological and clinical aspects of heme oxygenase. Pharmacol Rev. 60**:** 79-127.

Abrao, M.S., Dias, J.A., Jr., Bellelis, P., Podgaec, S., Bautzer, C.R. & Gromatsky, C. (2009). Endometriosis of the ureter and bladder are not associated diseases. Fertil Steril. 91**:** 1662-7.

Abrao, M.S., Neme, R.M., Carvalho, F.M., Aldrighi, J.M. & Pinotti, J.A. (2003). Histological classification of endometriosis as a predictor of response to treatment. Int J Gynaecol Obstet. 82**:** 31-40.

AFS (1979). Classification of endometriosis. The American Fertility Society. Fertil Steril. 32**:** 633-4.

AFS (1985). Revised American Fertility Society classification of endometriosis: 1985. Fertil Steril. 43**:** 351-2.

Agarwal, A., Gupta, S. & Sharma, R.K. (2005). Role of oxidative stress in female reproduction. Reprod Biol Endocrinol. 3**:** 28.

Agarwal, A., Saleh, R.A. & Bedaiwy, M.A. (2003). Role of reactive oxygen species in the pathophysiology of human reproduction. Fertil Steril. 79**:** 829-43.

Ailawadi, R.K., Jobanputra, S., Kataria, M., Gurates, B. & Bulun, S.E. (2004). Treatment of endometriosis and chronic pelvic pain with letrozole and norethindrone acetate: a pilot study. Fertil Steril. 81**:** 290-6.

Al-Gubory, K.H., Fowler, P.A. & Garrel, C. (2010). The roles of cellular reactive oxygen species, oxidative stress and antioxidants in pregnancy outcomes. Int J Biochem Cell Biol. 42**:** 1634-50.

Alao, J.P., Gamble, S.C., Stavropoulou, A.V., Pomeranz, K.M., Lam, E.W., Coombes, R.C. & Vigushin, D.M. (2006). The cyclin D1 proto-oncogene is sequestered in the cytoplasm of mammalian cancer cell lines. Mol Cancer. 5**:** 7.

Amer (2008). Endometriosis. Obstetrics, Gynaecology and Reproductive Medicine 18**:** 126-133.

Arumugam, K. (1994). Endometriosis and infertility: raised iron concentration in the peritoneal fluid and its effect on the acrosome reaction. Hum Reprod. 9**:** 1153-7.

Arumugam, K. & Dip, Y.C. (1995). Endometriosis and infertility: the role of exogenous lipid peroxides in the peritoneal fluid. Fertil Steril. 63**:** 198-9.

Aruoma, O.I., Halliwell, B., Hoey, B.M. & Butler, J. (1989). The antioxidant action of N-acetylcysteine: its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and hypochlorous acid. Free Radic Biol Med. 6**:** 593-7.

Arvanitis, D.A., Koumantakis, G.E., Goumenou, A.G., Matalliotakis, I.M., Koumantakis, E.E. & Spandidos, D.A. (2003). CYP1A1, CYP19, and GSTM1 polymorphisms increase the risk of endometriosis. Fertil Steril. 79 Suppl 1**:** 702-9.

ASRM (1997). Revised American Society for Reproductive Medicine classification of endometriosis: 1996. Fertil Steril. 67**:** 817-21.

ASRM (2008). Treatment of pelvic pain associated with endometriosis. Fertil Steril. 90**:** S260-9.

Atkuri, K.R., Mantovani, J.J., Herzenberg, L.A. & Herzenberg, L.A. (2007). N-Acetylcysteine--a safe antidote for cysteine/glutathione deficiency. Curr Opin Pharmacol. 7**:** 355-9.

Attar, E. & Bulun, S.E. (2006a). Aromatase and other steroidogenic genes in endometriosis: translational aspects. Hum Reprod Update. 12**:** 49-56.

Attar, E. & Bulun, S.E. (2006b). Aromatase inhibitors: the next generation of therapeutics for endometriosis? Fertil Steril. 85**:** 1307-18.

Bae, J.Y., Ahn, S.J., Han, W. & Noh, D.Y. (2007). Peroxiredoxin I and II inhibit H2O2-induced cell death in MCF-7 cell lines. J Cell Biochem. 101**:** 1038-45.

Balla, J., Nath, K.A., Balla, G., Juckett, M.B., Jacob, H.S. & Vercellotti, G.M. (1995). Endothelial cell heme oxygenase and ferritin induction in rat lung by hemoglobin in vivo. Am J Physiol. 268**:** L321-7.

Banu, S.K., Lee, J., Starzinski-Powitz, A. & Arosh, J.A. (2008). Gene expression profiles and functional characterization of human immortalized endometriotic epithelial and stromal cells. Fertil Steril. 90**:** 972-87.

Banu, S.K., Starzinski-Powitz, A., Speights, V.O., Burghardt, R.C. & Arosh, J.A. (2009). Induction of peritoneal endometriosis in nude mice with use of human immortalized endometriosis epithelial and stromal cells: a potential experimental tool to study molecular pathogenesis of endometriosis in humans. Fertil Steril. 91**:** 2199-209.

Bedaiwy, M.A. & Falcone, T. (2004). Laboratory testing for endometriosis. Clin Chim Acta. 340**:** 41-56.

Bedaiwy, M.A., Falcone, T., Sharma, R.K., Goldberg, J.M., Attaran, M., Nelson, D.R. & Agarwal, A. (2002). Prediction of endometriosis with serum and peritoneal fluid markers: a prospective controlled trial. Hum Reprod. 17**:** 426-31.

Behr, J., Maier, K., Degenkolb, B., Krombach, F. & Vogelmeier, C. (1997). Antioxidative and clinical effects of high-dose N-acetylcysteine in fibrosing alveolitis. Adjunctive therapy to maintenance immunosuppression. Am J Respir Crit Care Med. 156**:** 1897-901.

Belcher, S.M. & Zsarnovszky, A. (2001). Estrogenic actions in the brain: estrogen, phytoestrogens, and rapid intracellular signaling mechanisms. J Pharmacol Exp Ther. 299**:** 408-14.

Bennett, B.L., Sasaki, D.T., Murray, B.W., O'Leary, E.C., Sakata, S.T., Xu, W., Leisten, J.C., Motiwala, A., Pierce, S., Satoh, Y., Bhagwat, S.S., Manning, A.M. & Anderson, D.W. (2001). SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. Proc Natl Acad Sci U S A. 98**:** 13681-6.

Bergqvist, A., Ferno, M. & Skoog, L. (1997). Quantitative enzyme immunoassay and semiquantitative immunohistochemistry of oestrogen and progesterone receptors in endometriotic tissue and endometrium. J Clin Pathol. 50**:** 496-500.

Berk, M., Copolov, D., Dean, O., Lu, K., Jeavons, S., Schapkaitz, I., Anderson-Hunt, M., Judd, F., Katz, F., Katz, P., Ording-Jespersen, S., Little, J., Conus, P., Cuenod, M., Do, K.Q. & Bush, A.I. (2008). N-acetyl cysteine as a glutathione precursor for schizophrenia--a double-blind, randomized, placebo-controlled trial. Biol Psychiatry. 64**:** 361-8.

Blanc, A., Pandey, N.R. & Srivastava, A.K. (2003). Synchronous activation of ERK 1/2, p38mapk and PKB/Akt signaling by H2O2 in vascular smooth muscle cells: potential involvement in vascular disease (review). Int J Mol Med. 11**:** 229-34.

Bonanomi, L. & Gazzaniga, A. (1980). Toxicological, pharmacokinetic and metabolic studies on acetylcysteine. Eur J Respir Dis Suppl. 111**:** 45-51.

Borgstrom, L., Kagedal, B. & Paulsen, O. (1986). Pharmacokinetics of N-acetylcysteine in man. Eur J Clin Pharmacol. 31**:** 217-22.

Boxer, L.M. & Dang, C.V. (2001). Translocations involving c-myc and c-myc function. Oncogene. 20**:** 5595-610.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem. 72**:** 248-54.

Brandenberger, A.W., Lebovic, D.I., Tee, M.K., Ryan, I.P., Tseng, J.F., Jaffe, R.B. & Taylor, R.N. (1999). Oestrogen receptor (ER)-alpha and ER-beta isoforms in normal endometrial and endometriosis-derived stromal cells. Mol Hum Reprod. 5**:** 651-5.

Braun, D.P., Ding, J., Shen, J., Rana, N., Fernandez, B.B. & Dmowski, W.P. (2002). Relationship between apoptosis and the number of macrophages in eutopic endometrium from women with and without endometriosis. Fertil Steril. 78**:** 830-5.

Bricou, A., Batt, R.E. & Chapron, C. (2008). Peritoneal fluid flow influences anatomical distribution of endometriotic lesions: why Sampson seems to be right. Eur J Obstet Gynecol Reprod Biol. 138**:** 127-34.

Brosens, I., Puttemans, P. & Deprest, J. (1993). Appearances of endometriosis. Baillieres Clin Obstet Gynaecol. 7**:** 741-57.

Brosens, I.A. & Brosens, J.J. (2000). Endometriosis. Eur J Obstet Gynecol Reprod Biol. 90**:** 159-64.

Buder-Hoffmann, S., Palmer, C., Vacek, P., Taatjes, D. & Mossman, B. (2001). Different accumulation of activated extracellular signal-regulated kinases (ERK 1/2) and role in cell-cycle alterations by epidermal growth factor, hydrogen peroxide, or asbestos in pulmonary epithelial cells. Am J Respir Cell Mol Biol. 24**:** 405-13.

Bukulmez, O., Hardy, D.B., Carr, B.R., Word, R.A. & Mendelson, C.R. (2008). Inflammatory status influences aromatase and steroid receptor expression in endometriosis. Endocrinology. 149**:** 1190-204.

Bulletti, C., DeZiegler, D., Stefanetti, M., Cicinelli, E., Pelosi, E. & Flamigni, C. (2001). Endometriosis: absence of recurrence in patients after endometrial ablation. Hum Reprod. 16**:** 2676-9.

Bulun, S.E., Cheng, Y.H., Pavone, M.E., Xue, Q., Attar, E., Trukhacheva, E., Tokunaga, H., Utsunomiya, H., Yin, P., Luo, X., Lin, Z., Imir, G., Thung, S., Su, E.J. & Kim, J.J. (2010). Estrogen receptor-beta, estrogen receptor-alpha, and progesterone resistance in endometriosis. Semin Reprod Med. 28**:** 36-43.

Bulun, S.E., Cheng, Y.H., Yin, P., Imir, G., Utsunomiya, H., Attar, E., Innes, J. & Julie Kim, J. (2006). Progesterone resistance in endometriosis: link to failure to metabolize estradiol. Mol Cell Endocrinol. 248**:** 94-103.

Bulun, S.E., Gurates, B., Fang, Z., Tamura, M., Sebastian, S., Zhou, J., Amin, S. & Yang, S. (2002a). Mechanisms of excessive estrogen formation in endometriosis. J Reprod Immunol. 55**:** 21-33.

Bulun, S.E., Yang, S., Fang, Z., Gurates, B., Tamura, M. & Sebastian, S. (2002b). Estrogen production and metabolism in endometriosis. Ann N Y Acad Sci. 955**:** 75-85; discussion 86-8, 396-406.

Bulun, S.E., Zeitoun, K., Takayama, K., Noble, L., Michael, D., Simpson, E., Johns, A., Putman, M. & Sasano, H. (1999). Estrogen production in endometriosis and use of aromatase inhibitors to treat endometriosis. Endocr Relat Cancer. 6**:** 293-301.

Burney, R.O., Talbi, S., Hamilton, A.E., Vo, K.C., Nyegaard, M., Nezhat, C.R., Lessey, B.A. & Giudice, L.C. (2007). Gene expression analysis of endometrium reveals progesterone resistance and candidate susceptibility genes in women with endometriosis. Endocrinology. 148**:** 3814-26.

Carli, C., Metz, C.N., Al-Abed, Y., Naccache, P.H. & Akoum, A. (2009). Up-regulation of cyclooxygenase-2 expression and prostaglandin E2 production in human endometriotic cells by macrophage migration inhibitory factor: involvement of novel kinase signaling pathways. Endocrinology. 150**:** 3128-37.

Chen, G., Shi, J., Hu, Z. & Hang, C. (2008). Inhibitory effect on cerebral inflammatory response following traumatic brain injury in rats: a potential neuroprotective mechanism of N-acetylcysteine. Mediators Inflamm. 2008**:** 716458.

Chiao, J.W., Chung, F., Krzeminski, J., Amin, S., Arshad, R., Ahmed, T. & Conaway, C.C. (2000). Modulation of growth of human prostate cancer cells by the N-acetylcysteine conjugate of phenethyl isothiocyanate. Int J Oncol. 16**:** 1215-9.

Child, T.J. & Tan, S.L. (2001). Endometriosis: aetiology, pathogenesis and treatment. Drugs. 61**:** 1735-50.

Conde de la Rosa, L., Schoemaker, M.H., Vrenken, T.E., Buist-Homan, M., Havinga, R., Jansen, P.L. & Moshage, H. (2006) Superoxide anions and hydrogen peroxide induce hepatocyte death by different mechanisms: involvement of JNK and ERK MAP kinases. *J Hepatol.*

Cory, S. & Adams, J.M. (2002). The Bcl2 family: regulators of the cellular life-or-death switch. Nat Rev Cancer. 2**:** 647-56.

Cramer, D.W. & Missmer, S.A. (2002). The epidemiology of endometriosis. Ann N Y Acad Sci. 955**:** 11-22; discussion 34-6, 396-406.

Dassen, H., Punyadeera, C., Kamps, R., Delvoux, B., Van Langendonckt, A., Donnez, J., Husen, B., Thole, H., Dunselman, G. & Groothuis, P. (2007). Estrogen metabolizing enzymes in endometrium and endometriosis. Hum Reprod. 22**:** 3148-58.

Davis, R.J. (2000). Signal transduction by the JNK group of MAP kinases. Cell. 103**:** 239-52.

Day, J.M., Foster, P.A., Tutill, H.J., Parsons, M.F., Newman, S.P., Chander, S.K., Allan, G.M., Lawrence, H.R., Vicker, N., Potter, B.V., Reed, M.J. & Purohit, A. (2008). 17beta-hydroxysteroid dehydrogenase Type 1, and not Type 12, is a target for endocrine therapy of hormone-dependent breast cancer. Int J Cancer. 122**:** 1931-40.

De Rosa, S.C., Zaretsky, M.D., Dubs, J.G., Roederer, M., Anderson, M., Green, A., Mitra, D., Watanabe, N., Nakamura, H., Tjioe, I., Deresinski, S.C., Moore, W.A., Ela, S.W., Parks, D., Herzenberg, L.A. & Herzenberg, L.A. (2000). N-acetylcysteine replenishes glutathione in HIV infection. Eur J Clin Invest. 30**:** 915-29.

De Vries, N. & De Flora, S. (1993). N-acetyl-l-cysteine. J Cell Biochem Suppl. 17F**:** 270-7.

Defrere, S., Lousse, J.C., Gonzalez-Ramos, R., Colette, S., Donnez, J. & Van Langendonckt, A. (2008). Potential involvement of iron in the pathogenesis of peritoneal endometriosis. Mol Hum Reprod. 14**:** 377-85.

Defrere, S., Van Langendonckt, A., Vaesen, S., Jouret, M., Gonzalez Ramos, R., Gonzalez, D. & Donnez, J. (2006). Iron overload enhances epithelial cell proliferation in endometriotic lesions induced in a murine model. Hum Reprod. 21**:** 2810-6.

Deligdisch, L. (2000). Hormonal pathology of the endometrium. Mod Pathol. 13**:** 285-94.

Delvoux, B., Groothuis, P., D'Hooghe, T., Kyama, C., Dunselman, G. & Romano, A. (2009). Increased production of 17beta-estradiol in endometriosis lesions is the result of impaired metabolism. J Clin Endocrinol Metab. 94**:** 876-83.

Demedts, I.K., Demoor, T., Bracke, K.R., Joos, G.F. & Brusselle, G.G. (2006). Role of apoptosis in the pathogenesis of COPD and pulmonary emphysema. Respir Res. 7**:** 53.

Dickinson, D.A. & Forman, H.J. (2002). Cellular glutathione and thiols metabolism. Biochem Pharmacol. 64**:** 1019-26.

Dickinson, D.A., Moellering, D.R., Iles, K.E., Patel, R.P., Levonen, A.L., Wigley, A., Darley-Usmar, V.M. & Forman, H.J. (2003). Cytoprotection against oxidative stress and the regulation of glutathione synthesis. Biol Chem. 384**:** 527-37.

Dmowski, W.P., Ding, J., Shen, J., Rana, N., Fernandez, B.B. & Braun, D.P. (2001). Apoptosis in endometrial glandular and stromal cells in women with and without endometriosis. Hum Reprod. 16**:** 1802-8.

Dong, Z., Lavrovsky, Y., Venkatachalam, M.A. & Roy, A.K. (2000). Heme oxygenase-1 in tissue pathology: the Yin and Yang. Am J Pathol. 156**:** 1485-8.

Dragin, N., Smani, M., Arnaud-Dabernat, S., Dubost, C., Moranvillier, I., Costet, P., Daniel, J.Y. & Peuchant, E. (2006). Acute oxidative stress is associated with cell proliferation in the mouse liver. FEBS Lett. 580**:** 3845-52.

Eady, J.J., Orta, T., Dennis, M.F., Stratford, M.R. & Peacock, J.H. (1995). Glutathione determination by the Tietze enzymatic recycling assay and its relationship to cellular radiation response. Br J Cancer. 72**:** 1089-95.

Esterbauer, H., Schaur, R.J. & Zollner, H. (1991). Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. Free Radic Biol Med. 11**:** 81-128.

Fazleabas, A.T., Brudney, A., Chai, D., Langoi, D. & Bulun, S.E. (2003). Steroid receptor and aromatase expression in baboon endometriotic lesions. Fertil Steril. 80 Suppl 2**:** 820-7.

Fazleabas, A.T., Brudney, A., Gurates, B., Chai, D. & Bulun, S. (2002). A modified baboon model for endometriosis. Ann N Y Acad Sci. 955**:** 308-17; discussion 340-2, 396-406.

Fishbane, S., Durham, J.H., Marzo, K. & Rudnick, M. (2004). N-acetylcysteine in the prevention of radiocontrast-induced nephropathy. J Am Soc Nephrol. 15**:** 251-60.

Fitzgerald, K.A., McWhirter, S.M., Faia, K.L., Rowe, D.C., Latz, E., Golenbock, D.T., Coyle, A.J., Liao, S.M. & Maniatis, T. (2003). IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. Nat Immunol. 4**:** 491-6.

Flores, I., Abreu, S., Abac, S., Fourquet, J., Laboy, J. & Rios-Bedoya, C. (2008). Self-reported prevalence of endometriosis and its symptoms among Puerto Rican women. Int J Gynaecol Obstet. 100**:** 257-61.

Flores, I., Rivera, E., Ruiz, L.A., Santiago, O.I., Vernon, M.W. & Appleyard, C.B. (2007). Molecular profiling of experimental endometriosis identified gene expression patterns in common with human disease. Fertil Steril. 87**:** 1180-99.

Forman, H.J., Zhang, H. & Rinna, A. (2009). Glutathione: overview of its protective roles, measurement, and biosynthesis. Mol Aspects Med. 30**:** 1-12.

Foster, J.S., Henley, D.C., Ahamed, S. & Wimalasena, J. (2001). Estrogens and cell-cycle regulation in breast cancer. Trends Endocrinol Metab. 12**:** 320-7.

Fotakis, G. & Timbrell, J.A. (2006). In vitro cytotoxicity assays: comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. Toxicol Lett. 160**:** 171-7.

Foyouzi, N., Berkkanoglu, M., Arici, A., Kwintkiewicz, J., Izquierdo, D. & Duleba, A.J. (2004). Effects of oxidants and antioxidants on proliferation of endometrial stromal cells. Fertil Steril. 82 Suppl 3**:** 1019-22.

Frenkel, K. (1992). Carcinogen-mediated oxidant formation and oxidative DNA damage. Pharmacol Ther. 53**:** 127-66.

Galluzzi, L., Zamzami, N., de La Motte Rouge, T., Lemaire, C., Brenner, C. & Kroemer, G. (2007). Methods for the assessment of mitochondrial membrane permeabilization in apoptosis. Apoptosis. 12**:** 803-13.

Gebel, H.M., Braun, D.P., Tambur, A., Frame, D., Rana, N. & Dmowski, W.P. (1998). Spontaneous apoptosis of endometrial tissue is impaired in women with endometriosis. Fertil Steril. 69**:** 1042-7.

Ghosh, D., Griswold, J., Erman, M. & Pangborn, W. (2009). Structural basis for androgen specificity and oestrogen synthesis in human aromatase. Nature. 457**:** 219-23.

Giudice, L.C. & Kao, L.C. (2004). Endometriosis. Lancet. 364**:** 1789-99.

Gomes, M.K., Rosa-e-Silva, J.C., Garcia, S.B., de Sa Rosa-e-Silva, A.C., Turatti, A., Vieira, C.S. & Ferriani, R.A. (2009). Effects of the levonorgestrel-releasing intrauterine system on cell proliferation, Fas expression and steroid receptors in endometriosis lesions and normal endometrium. Hum Reprod. 24**:** 2736-45.

Gopinathan, V., Miller, N.J., Milner, A.D. & Rice-Evans, C.A. (1994). Bilirubin and ascorbate antioxidant activity in neonatal plasma. FEBS Lett. 349**:** 197-200.

Graca-Souza, A.V., Maya-Monteiro, C., Paiva-Silva, G.O., Braz, G.R., Paes, M.C., Sorgine, M.H., Oliveira, M.F. & Oliveira, P.L. (2006). Adaptations against heme toxicity in blood-feeding arthropods. Insect Biochem Mol Biol. 36**:** 322-35.

Gross, A., McDonnell, J.M. & Korsmeyer, S.J. (1999). BCL-2 family members and the mitochondria in apoptosis. Genes Dev. 13**:** 1899-911.

Grund, E.M., Kagan, D., Tran, C.A., Zeitvogel, A., Starzinski-Powitz, A., Nataraja, S. & Palmer, S.S. (2008). Tumor necrosis factor-alpha regulates inflammatory and mesenchymal responses via mitogen-activated protein kinase kinase, p38, and nuclear factor kappaB in human endometriotic epithelial cells. Mol Pharmacol. 73**:** 1394-404.

Gupta, M.K., Neelakantan, T.V., Sanghamitra, M., Tyagi, R.K., Dinda, A., Maulik, S., Mukhopadhyay, C.K. & Goswami, S.K. (2006). An assessment of the role of reactive oxygen species and redox signaling in norepinephrine-induced apoptosis and hypertrophy of H9c2 cardiac myoblasts. Antioxid Redox Signal. 8**:** 1081-93.

Gutteridge, J.M. & Smith, A. (1988). Antioxidant protection by haemopexin of haem-stimulated lipid peroxidation. Biochem J. 256**:** 861-5.

Guzeloglu Kayisli, O., Kayisli, U.A., Luleci, G. & Arici, A. (2004). In vivo and in vitro regulation of Akt activation in human endometrial cells is estrogen dependent. Biol Reprod. 71**:** 714-21.

Hadfield, R.M., Mardon, H.J., Barlow, D.H. & Kennedy, S.H. (1997). Endometriosis in monozygotic twins. Fertil Steril. 68**:** 941-2.

Halis, G., Mechsner, S. & Ebert, A.D. (2010). The diagnosis and treatment of deep infiltrating endometriosis. Dtsch Arztebl Int. 107**:** 446-55; quiz 456.

Halliwell, B. (1987). Oxidants and human disease: some new concepts. Faseb J. 1**:** 358-64.

Halliwell, B. (1994). Free radicals, antioxidants, and human disease: curiosity, cause, or consequence? Lancet. 344**:** 721-4.

Halliwell, B. (2006). Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. Plant Physiol. 141**:** 312-22.

Halliwell, B. (2007). Biochemistry of oxidative stress. Biochem Soc Trans. 35**:** 1147-50.

Halliwell, B. & Whiteman, M. (2004). Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? Br J Pharmacol. 142**:** 231-55.

Halme, J., Hammond, M.G., Hulka, J.F., Raj, S.G. & Talbert, L.M. (1984). Retrograde menstruation in healthy women and in patients with endometriosis. Obstet Gynecol. 64**:** 151-4.

Haney, A.F., Muscato, J.J. & Weinberg, J.B. (1981). Peritoneal fluid cell populations in infertility patients. Fertil Steril. 35**:** 696-8.

Hansen, R.J., Ludeman, S.M., Paikoff, S.J., Pegg, A.E. & Dolan, M.E. (2007). Role of MGMT in protecting against cyclophosphamide-induced toxicity in cells and animals. DNA Repair (Amst). 6**:** 1145-54.

Harada, T., Iwabe, T. & Terakawa, N. (2001). Role of cytokines in endometriosis. Fertil Steril. 76**:** 1-10.

Harada, T., Kaponis, A., Iwabe, T., Taniguchi, F., Makrydimas, G., Sofikitis, N., Paschopoulos, M., Paraskevaidis, E. & Terakawa, N. (2004). Apoptosis in human endometrium and endometriosis. Hum Reprod Update. 10**:** 29-38.

Harris, J., Oliere, S., Sharma, S., Sun, Q., Lin, R., Hiscott, J. & Grandvaux, N. (2006). Nuclear accumulation of cRel following C-terminal phosphorylation by TBK1/IKK epsilon. J Immunol. 177**:** 2527-35.

Hasbi, A., O'Dowd, B.F. & George, S.R. (2005). A G protein-coupled receptor for estrogen: the end of the search? Mol Interv. 5**:** 158-61.

Hastings, J.M. & Fazleabas, A.T. (2006). A baboon model for endometriosis: implications for fertility. Reprod Biol Endocrinol. 4 Suppl 1**:** S7.

Heidebrecht, F., Heidebrecht, A., Schulz, I., Behrens, S.E. & Bader, A. (2009). Improved semiquantitative Western blot technique with increased quantification range. J Immunol Methods. 345**:** 40-8.

Heilier, J.F., Donnez, O., Van Kerckhove, V., Lison, D. & Donnez, J. (2006). Expression of aromatase (P450 aromatase/CYP19) in peritoneal and ovarian endometriotic tissues and deep endometriotic (adenomyotic) nodules of the rectovaginal septum. Fertil Steril. 85**:** 1516-8.

Hernandez-Garcia, D., Wood, C.D., Castro-Obregon, S. & Covarrubias, L. (2010). Reactive oxygen species: A radical role in development? Free Radic Biol Med. 49**:** 130-43.

Ho, H.N., Wu, M.Y., Chen, S.U., Chao, K.H., Chen, C.D. & Yang, Y.S. (1997). Total antioxidant status and nitric oxide do not increase in peritoneal fluids from women with endometriosis. Hum Reprod. 12**:** 2810-5.

Honda, H., Barrueto, F.F., Gogusev, J., Im, D.D. & Morin, P.J. (2008). Serial analysis of gene expression reveals differential expression between endometriosis and normal endometrium. Possible roles for AXL and SHC1 in the pathogenesis of endometriosis. Reprod Biol Endocrinol. 6**:** 59.

Huang, H.Y. (2008). Medical treatment of endometriosis. Chang Gung Med J. 31**:** 431-40.

Hudelist, G., Keckstein, J., Czerwenka, K., Lass, H., Walter, I., Auer, M., Wieser, F., Wenzl, R., Kubista, E. & Singer, C.F. (2005). Estrogen receptor beta and matrix metalloproteinase 1 are coexpressed in uterine endometrium and endometriotic lesions of patients with endometriosis. Fertil Steril. 84 Suppl 2**:** 1249-56.

Hurd, W.W. (1998). Criteria that indicate endometriosis is the cause of chronic pelvic pain. Obstet Gynecol. 92**:** 1029-32.

Imai, A., Takagi, A. & Tamaya, T. (2000). Gonadotropin-releasing hormone analog repairs reduced endometrial cell apoptosis in endometriosis in vitro. Am J Obstet Gynecol. 182**:** 1142-6.

Izawa, M., Harada, T., Taniguchi, F., Ohama, Y., Takenaka, Y. & Terakawa, N. (2008). An epigenetic disorder may cause aberrant expression of aromatase gene in endometriotic stromal cells. Fertil Steril. 89**:** 1390-6.

Izawa, S., Inoue, Y. & Kimura, A. (1995). Oxidative stress response in yeast: effect of glutathione on adaptation to hydrogen peroxide stress in Saccharomyces cerevisiae. FEBS Lett. 368**:** 73-6.

Jackson, L.W., Schisterman, E.F., Dey-Rao, R., Browne, R. & Armstrong, D. (2005). Oxidative stress and endometriosis. Hum Reprod. 20**:** 2014-20.

Jones, C.M., Lawrence, A., Wardman, P. & Burkitt, M.J. (2003). Kinetics of superoxide scavenging by glutathione: an evaluation of its role in the removal of mitochondrial superoxide. Biochem Soc Trans. 31**:** 1337-9.

Karlsson, H., Nava, S., Remberger, M., Hassan, Z., Hassan, M. & Ringden, O. (2011). N-acetyl-L-cysteine increases acute graft-versus-host disease and promotes T-cell-mediated immunity in vitro. Eur J Immunol.

Kashima, K., Ishimaru, T., Okamura, H., Suginami, H., Ikuma, K., Murakami, T., Iwashita, M. & Tanaka, K. (2004). Familial risk among Japanese patients with endometriosis. Int J Gynaecol Obstet. 84**:** 61-4.

Kaspar, J.W., Niture, S.K. & Jaiswal, A.K. (2009). Nrf2:INrf2 (Keap1) signaling in oxidative stress. Free Radic Biol Med. 47**:** 1304-9.

Katzenellenbogen, B.S. (1996). Estrogen receptors: bioactivities and interactions with cell signaling pathways. Biol Reprod. 54**:** 287-93.

Katzenellenbogen, B.S., Choi, I., Delage-Mourroux, R., Ediger, T.R., Martini, P.G., Montano, M., Sun, J., Weis, K. & Katzenellenbogen, J.A. (2000). Molecular mechanisms of estrogen action: selective ligands and receptor pharmacology. J Steroid Biochem Mol Biol. 74**:** 279-85.

Kennedy, S., Bergqvist, A., Chapron, C., D'Hooghe, T., Dunselman, G., Greb, R., Hummelshoj, L., Prentice, A. & Saridogan, E. (2005). ESHRE guideline for the diagnosis and treatment of endometriosis. Hum Reprod. 20**:** 2698-704.

Kim, H.P., Pae, H.O., Back, S.H., Chung, S.W., Woo, J.M., Son, Y. & Chung, H.T. (2011). Heme oxygenase-1 comes back to endoplasmic reticulum. Biochem Biophys Res Commun. 404**:** 1-5.

Kinscherf, R., Fischbach, T., Mihm, S., Roth, S., Hohenhaus-Sievert, E., Weiss, C., Edler, L., Bartsch, P. & Droge, W. (1994). Effect of glutathione depletion and oral N-acetyl-cysteine treatment on CD4+ and CD8+ cells. Faseb J. 8**:** 448-51.

Kitawaki, J., Noguchi, T., Amatsu, T., Maeda, K., Tsukamoto, K., Yamamoto, T., Fushiki, S., Osawa, Y. & Honjo, H. (1997). Expression of aromatase cytochrome P450 protein and messenger ribonucleic acid in human endometriotic and adenomyotic tissues but not in normal endometrium. Biol Reprod. 57**:** 514-9.

Klaunig, J.E., Xu, Y., Isenberg, J.S., Bachowski, S., Kolaja, K.L., Jiang, J., Stevenson, D.E. & Walborg, E.F., Jr. (1998). The role of oxidative stress in chemical carcinogenesis. Environ Health Perspect. 106 Suppl 1**:** 289-95.

Klemmt, P.A., Carver, J.G., Koninckx, P., McVeigh, E.J. & Mardon, H.J. (2007). Endometrial cells from women with endometriosis have increased adhesion and proliferative capacity in response to extracellular matrix components: towards a mechanistic model for endometriosis progression. Hum Reprod. 22**:** 3139-47.

Klinge, C.M. (2001). Estrogen receptor interaction with estrogen response elements. Nucleic Acids Res. 29**:** 2905-19.

Ko, C.H., Shen, S.C., Hsu, C.S. & Chen, Y.C. (2005). Mitochondrial-dependent, reactive oxygen species-independent apoptosis by myricetin: roles of protein kinase C, cytochrome c, and caspase cascade. Biochem Pharmacol. 69**:** 913-27.

Kobayashi, H., Gil-Guzman, E., Mahran, A.M., Rakesh, Nelson, D.R., Thomas, A.J., Jr. & Agarwa, A. (2001). Quality control of reactive oxygen species measurement by luminol-dependent chemiluminescence assay. J Androl. 22**:** 568-74.

Kolls, J.K. (2006). Oxidative stress in sepsis: a redox redux. J Clin Invest. 116**:** 860-3.

Konno, R., Fujiwara, H., Netsu, S., Odagiri, K., Shimane, M., Nomura, H. & Suzuki, M. (2007). Gene expression profiling of the rat endometriosis model. Am J Reprod Immunol. 58**:** 330-43.

Korherr, C., Gille, H., Schafer, R., Koenig-Hoffmann, K., Dixelius, J., Egland, K.A., Pastan, I. & Brinkmann, U. (2006). Identification of proangiogenic genes and pathways by high-throughput functional genomics: TBK1 and the IRF3 pathway. Proc Natl Acad Sci U S A. 103**:** 4240-5.

Krauss, G. (2003). *Biochemistry of signal transduction and regulation,* Weinheim ; [Great Britain], Wiley-VCH.

Kyama, C.M., Overbergh, L., Mihalyi, A., Meuleman, C., Mwenda, J.M., Mathieu, C. & D'Hooghe, T.M. (2008). Endometrial and peritoneal expression of aromatase, cytokines, and adhesion factors in women with endometriosis. Fertil Steril. 89**:** 301-10.

Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 227**:** 680-5.

Lamb, K., Hoffmann, R.G. & Nichols, T.R. (1986). Family trait analysis: a case-control study of 43 women with endometriosis and their best friends. Am J Obstet Gynecol. 154**:** 596-601.

Lambrinoudaki, I.V., Augoulea, A., Christodoulakos, G.E., Economou, E.V., Kaparos, G., Kontoravdis, A., Papadias, C. & Creatsas, G. (2009). Measurable serum markers of oxidative stress response in women with endometriosis. Fertil Steril. 91**:** 46-50.

Lecce, G., Meduri, G., Ancelin, M., Bergeron, C. & Perrot-Applanat, M. (2001). Presence of estrogen receptor beta in the human endometrium through the cycle: expression in glandular, stromal, and vascular cells. J Clin Endocrinol Metab. 86**:** 1379-86.

Lee, Y.S., Yoon, H.J., Oh, J.H., Park, H.J., Lee, E.H., Song, C.W. & Yoon, S. (2009). 1,3-Dinitrobenzene induces apoptosis in TM4 mouse Sertoli cells: Involvement of the c-Jun N-terminal kinase (JNK) MAPK pathway. Toxicol Lett. 189**:** 145-51.

Lefloch, R., Pouyssegur, J. & Lenormand, P. (2008). Single and combined silencing of ERK1 and ERK2 reveals their positive contribution to growth signaling depending on their expression levels. Mol Cell Biol. 28**:** 511-27.

Lei, K., Nimnual, A., Zong, W.X., Kennedy, N.J., Flavell, R.A., Thompson, C.B., Bar-Sagi, D. & Davis, R.J. (2002). The Bax subfamily of Bcl2-related proteins is essential for apoptotic signal transduction by c-Jun NH(2)-terminal kinase. Mol Cell Biol. 22**:** 4929-42.

Liao, Y. & Hung, M.C. (2010). Physiological regulation of Akt activity and stability. Am J Transl Res. 2**:** 19-42.

Lin, B.C. & Scanlan, T.S. (2005). Few things in life are "free": cellular uptake of steroid hormones by an active transport mechanism. Mol Interv. 5**:** 338-40.

Lin, B.C., Suzawa, M., Blind, R.D., Tobias, S.C., Bulun, S.E., Scanlan, T.S. & Ingraham, H.A. (2009). Stimulating the GPR30 estrogen receptor with a novel tamoxifen analogue activates SF-1 and promotes endometrial cell proliferation. Cancer Res. 69**:** 5415-23.

Liu-Snyder, P., McNally, H., Shi, R. & Borgens, R.B. (2006). Acrolein-mediated mechanisms of neuronal death. J Neurosci Res. 84**:** 209-18.

Liu, J. & Lin, A. (2005). Role of JNK activation in apoptosis: a double-edged sword. Cell Res. 15**:** 36-42.

Liu, Y., Hu, J., Shen, W., Wang, J., Chen, C., Han, J., Zai, D., Cai, Z. & Yu, C. (2011). Peritoneal fluid of patients with endometriosis promotes proliferation of endometrial stromal cells and induces COX-2 expression. Fertil Steril. 95**:** 1836-8.

Liu, Y.W., Sakaeda, T., Takara, K., Nakamura, T., Ohmoto, N., Komoto, C., Kobayashi, H., Yagami, T., Okamura, N. & Okumura, K. (2003). Effects of reactive oxygen species on cell proliferation and death in HeLa cells and its MDR1-overexpressing derivative cell line. Biol Pharm Bull. 26**:** 278-81.

Long, X., Gize, E.A., Nephew, K. & Bigsby, R.M. (2001). Evidence for estrogenic contamination of the MAPK inhibitor PD98059. Endocrinology. 142**:** 5390-3.

Luciano, A.A., Turksoy, R.N. & Carleo, J. (1988). Evaluation of oral medroxyprogesterone acetate in the treatment of endometriosis. Obstet Gynecol. 72**:** 323-7.

Luo, X., Ding, L. & Chegini, N. (2004). Gonadotropin-releasing hormone and TGF-beta activate MAP kinase and differentially regulate fibronectin expression in endometrial epithelial and stromal cells. Am J Physiol Endocrinol Metab. 287**:** E991-E1001.

Maheshwari, A., Misro, M.M., Aggarwal, A., Sharma, R.K. & Nandan, D. (2011). N-acetyl-L-cysteine counteracts oxidative stress and prevents H2O2 induced germ cell apoptosis through down-regulation of caspase-9 and JNK/c-Jun. Mol Reprod Dev. 78**:** 69-79.

Mahmood, T.A. & Templeton, A. (1991). Prevalence and genesis of endometriosis. Hum Reprod. 6**:** 544-9.

Mahutte, N.G. & Arici, A. (2002). New advances in the understanding of endometriosis related infertility. J Reprod Immunol. 55**:** 73-83.

Mahutte, N.G. & Arici, A. (2003). Medical management of endometriosis-associated pain. Obstet Gynecol Clin North Am. 30**:** 133-50.

Mangelsdorf, D.J., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P. & Evans, R.M. (1995). The nuclear receptor superfamily: the second decade. Cell. 83**:** 835-9.

Marino, M., Galluzzo, P. & Ascenzi, P. (2006). Estrogen signaling multiple pathways to impact gene transcription. Curr Genomics. 7**:** 497-508.

Marjoribanks, J., Proctor, M., Farquhar, C. & Derks, R.S. (2010). Nonsteroidal anti-inflammatory drugs for dysmenorrhoea. Cochrane Database Syst Rev. CD001751.

Marnett, L.J. (2000). Oxyradicals and DNA damage. Carcinogenesis. 21**:** 361-70.

Matalliotakis, I.M., Cakmak, H., Fragouli, Y.G., Goumenou, A.G., Mahutte, N.G. & Arici, A. (2008). Epidemiological characteristics in women with and without endometriosis in the Yale series. Arch Gynecol Obstet. 277**:** 389-93.

Matalliotakis, I.M., Cakmak, H., Krasonikolakis, G.D., Dermitzaki, D., Fragouli, Y., Vlastos, G. & Arici, A. (2009). Endometriosis related to family history of malignancies in the Yale series. Surg Oncol.

Matalliotakis, I.M., Cakmak, H., Mahutte, N., Fragouli, Y., Arici, A. & Sakkas, D. (2007). Women with advanced-stage endometriosis and previous surgery respond less well to gonadotropin stimulation, but have similar IVF implantation and delivery rates compared with women with tubal factor infertility. Fertil Steril. 88**:** 1568-72.

Matorras, R., Rodiquez, F., Pijoan, J.I., Ramon, O., Gutierrez de Teran, G. & Rodriguez-Escudero, F. (1995). Epidemiology of endometriosis in infertile women. Fertil Steril. 63**:** 34-8.

Matsuzaki, S., Canis, M., Pouly, J.L., Dechelotte, P.J. & Mage, G. (2006). Analysis of aromatase and 17beta-hydroxysteroid dehydrogenase type 2 messenger

ribonucleic acid expression in deep endometriosis and eutopic endometrium using laser capture microdissection. Fertil Steril. 85**:** 308-13.

Matsuzaki, S., Fukaya, T., Uehara, S., Murakami, T., Sasano, H. & Yajima, A. (2000). Characterization of messenger RNA expression of estrogen receptor-alpha and -beta in patients with ovarian endometriosis. Fertil Steril. 73**:** 1219-25.

Matsuzaki, S., Murakami, T., Uehara, S., Canis, M., Sasano, H. & Okamura, K. (2001). Expression of estrogen receptor alpha and beta in peritoneal and ovarian endometriosis. Fertil Steril. 75**:** 1198-205.

McCoubrey, W.K., Jr., Huang, T.J. & Maines, M.D. (1997). Isolation and characterization of a cDNA from the rat brain that encodes hemoprotein heme oxygenase-3. Eur J Biochem. 247**:** 725-32.

McCubrey, J.A., Steelman, L.S., Abrams, S.L., Lee, J.T., Chang, F., Bertrand, F.E., Navolanic, P.M., Terrian, D.M., Franklin, R.A., D'Assoro, A.B., Salisbury, J.L., Mazzarino, M.C., Stivala, F. & Libra, M. (2006). Roles of the RAF/MEK/ERK and PI3K/PTEN/AKT pathways in malignant transformation and drug resistance. Adv Enzyme Regul. 46**:** 249-79.

Meresman, G.F., Auge, L., Baranao, R.I., Lombardi, E., Tesone, M. & Sueldo, C. (2002). Oral contraceptives suppress cell proliferation and enhance apoptosis of eutopic endometrial tissue from patients with endometriosis. Fertil Steril. 77**:** 1141-7.

Mier-Cabrera, J., Aburto-Soto, T., Burrola-Mendez, S., Jimenez-Zamudio, L., Tolentino, M.C., Casanueva, E. & Hernandez-Guerrero, C. (2009). Women with endometriosis improved their peripheral antioxidant markers after the application of a high antioxidant diet. Reprod Biol Endocrinol. 7**:** 54.

Mockett, R.J., Orr, W.C., Rahmandar, J.J., Benes, J.J., Radyuk, S.N., Klichko, V.I. & Sohal, R.S. (1999). Overexpression of Mn-containing superoxide dismutase in transgenic Drosophila melanogaster. Arch Biochem Biophys. 371**:** 260-9.

Moggs, J.G. & Orphanides, G. (2001). Estrogen receptors: orchestrators of pleiotropic cellular responses. EMBO Rep. 2**:** 775-81.

Mor, G., Straszewski, S. & Kamsteeg, M. (2002). Role of the Fas/Fas ligand system in female reproductive organs: survival and apoptosis. Biochem Pharmacol. 64**:** 1305-15.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 65**:** 55-63.

Moss, J.I., Pontes, E. & Hansen, P.J. (2009). Insulin-like growth factor-1 protects preimplantation embryos from anti-developmental actions of menadione. Arch Toxicol.

Murk, W., Atabekoglu, C.S., Cakmak, H., Heper, A., Ensari, A., Kayisli, U.A. & Arici, A. (2008). Extracellularly signal-regulated kinase activity in the human endometrium: possible roles in the pathogenesis of endometriosis. J Clin Endocrinol Metab. 93**:** 3532-40.

Murphy, A.A., Santanam, N. & Parthasarathy, S. (1998). Endometriosis: a disease of oxidative stress? Semin Reprod Endocrinol. 16**:** 263-73.

Muzio, M., Stockwell, B.R., Stennicke, H.R., Salvesen, G.S. & Dixit, V.M. (1998). An induced proximity model for caspase-8 activation. J Biol Chem. 273**:** 2926-30.

Nair, U., Bartsch, H. & Nair, J. (2007). Lipid peroxidation-induced DNA damage in cancer-prone inflammatory diseases: a review of published adduct types and levels in humans. Free Radic Biol Med. 43**:** 1109-20.

Nakago, S., Hadfield, R.M., Zondervan, K.T., Mardon, H., Manek, S., Weeks, D.E., Barlow, D. & Kennedy, S. (2001). Association between endometriosis and N-acetyl transferase 2 polymorphisms in a UK population. Mol Hum Reprod. 7**:** 1079-83.

Nap, A.W., Groothuis, P.G., Demir, A.Y., Evers, J.L. & Dunselman, G.A. (2004). Pathogenesis of endometriosis. Best Pract Res Clin Obstet Gynaecol. 18**:** 233-44.

Nascimento, M.M., Suliman, M.E., Silva, M., Chinaglia, T., Marchioro, J., Hayashi, S.Y., Riella, M.C., Lindholm, B. & Anderstam, B. (2010). Effect of oral N-acetylcysteine treatment on plasma inflammatory and oxidative stress markers in peritoneal dialysis patients: a placebo-controlled study. Perit Dial Int. 30**:** 336-42.

Nathan, C.F. (1987). Secretory products of macrophages. J Clin Invest. 79**:** 319-26.

Nawathe, A., Patwardhan, S., Yates, D., Harrison, G.R. & Khan, K.S. (2008). Systematic review of the effects of aromatase inhibitors on pain associated with endometriosis. Bjog. 115**:** 818-22.

Ngo, C., Chereau, C., Nicco, C., Weill, B., Chapron, C. & Batteux, F. (2009). Reactive oxygen species controls endometriosis progression. Am J Pathol. 175**:** 225-34.

Nguyen, T., Nioi, P. & Pickett, C.B. (2009). The Nrf2-antioxidant response element signaling pathway and its activation by oxidative stress. J Biol Chem. 284**:** 13291-5.

Nhien, N.T., Huy, N.T., Naito, M., Oida, T., Uyen, D.T., Huang, M., Kikuchi, M., Harada, S., Nakayama, K., Hirayama, K. & Kamei, K. (2010). Neutralization of toxic haem by Porphyromonas gingivalis haemoglobin receptor. J Biochem. 147**:** 317-25.

Noble, L.S., Takayama, K., Zeitoun, K.M., Putman, J.M., Johns, D.A., Hinshelwood, M.M., Agarwal, V.R., Zhao, Y., Carr, B.R. & Bulun, S.E. (1997). Prostaglandin E2 stimulates aromatase expression in endometriosis-derived stromal cells. J Clin Endocrinol Metab. 82**:** 600-6.

Noel, C.T., Reed, M.J., Jacobs, H.S. & James, V.H. (1981). The plasma concentration of oestrone sulphate in postmenopausal women: lack of diurnal variation, effect of ovariectomy, age and weight. J Steroid Biochem. 14**:** 1101-5.

Noordin, L., San, G.T., Singh, H.J., Othman, M.S. & Hafizah, W. (2008). Pyruvate reduces in vitro the embryotoxic effect of peritoneal fluid from infertile women with endometriosis. Eur J Obstet Gynecol Reprod Biol. 136**:** 67-73.

O'Callaghan, D. (2006). Endometriosis--an update. Aust Fam Physician. 35**:** 864-7.

Oral, E., Olive, D.L. & Arici, A. (1996). The peritoneal environment in endometriosis. Hum Reprod Update. 2**:** 385-98.

Osteen, K.G., Bruner-Tran, K.L. & Eisenberg, E. (2005). Endometrial biology and the etiology of endometriosis. Fertil Steril. 84**:** 33-4; discussion 38-9.

Ota, H., Igarashi, S., Hatazawa, J. & Tanaka, T. (1999a). Endometriosis and free radicals. Gynecol Obstet Invest. 48 Suppl 1**:** 29-35.

Ota, H., Igarashi, S., Hatazawa, J. & Tanaka, T. (1999b). Immunohistochemical assessment of superoxide dismutase expression in the endometrium in endometriosis and adenomyosis. Fertil Steril. 72**:** 129-34.

Ota, H., Igarashi, S. & Tanaka, T. (2001). Xanthine oxidase in eutopic and ectopic endometrium in endometriosis and adenomyosis. Fertil Steril. 75**:** 785-90.

Otterbein, L.E. & Choi, A.M. (2000). Heme oxygenase: colors of defense against cellular stress. Am J Physiol Lung Cell Mol Physiol. 279**:** L1029-37.

Ou, Y.H., Torres, M., Ram, R., Formstecher, E., Roland, C., Cheng, T., Brekken, R., Wurz, R., Tasker, A., Polverino, T., Tan, S.L. & White, M.A. (2011). TBK1 directly engages Akt/PKB survival signaling to support oncogenic transformation. Mol Cell. 41**:** 458-70.

Ozkan, S., Murk, W. & Arici, A. (2008). Endometriosis and infertility: epidemiology and evidence-based treatments. Ann N Y Acad Sci. 1127**:** 92-100.

Panahian, N., Yoshiura, M. & Maines, M.D. (1999). Overexpression of heme oxygenase-1 is neuroprotective in a model of permanent middle cerebral artery occlusion in transgenic mice. J Neurochem. 72**:** 1187-203.

Patwardhan, A., Griffiths, A. & Penketh, R. (2008). Surgical outcome and long-term follow up after laparoscopic rectosigmoid resection. Bjog. 115**:** 414; author reply 414-5.

Pearce, S.T. & Jordan, V.C. (2004). The biological role of estrogen receptors alpha and beta in cancer. Crit Rev Oncol Hematol. 50**:** 3-22.

Penning, T.M., Burczynski, M.E., Jez, J.M., Hung, C.F., Lin, H.K., Ma, H., Moore, M., Palackal, N. & Ratnam, K. (2000). Human 3alpha-hydroxysteroid dehydrogenase isoforms (AKR1C1-AKR1C4) of the aldo-keto reductase superfamily: functional plasticity and tissue distribution reveals roles in the inactivation and formation of male and female sex hormones. Biochem J. 351**:** 67-77.

Peskin, A.V. & Winterbourn, C.C. (2000). A microtiter plate assay for superoxide dismutase using a water-soluble tetrazolium salt (WST-1). Clin Chim Acta. 293**:** 157-66.

Pettersson, K. & Gustafsson, J.A. (2001). Role of estrogen receptor beta in estrogen action. Annu Rev Physiol. 63**:** 165-92.

Pittaluga, E., Costa, G., Krasnowska, E., Brunelli, R., Lundeberg, T., Porpora, M.G., Santucci, D. & Parasassi, T. (2010). More than antioxidant: N-acetyl-L-cysteine in a murine model of endometriosis. Fertil Steril. 94**:** 2905-8.

Pocernich, C.B., Cardin, A.L., Racine, C.L., Lauderback, C.M. & Butterfield, D.A. (2001). Glutathione elevation and its protective role in acrolein-induced protein damage in synaptosomal membranes: relevance to brain lipid peroxidation in neurodegenerative disease. Neurochem Int. 39**:** 141-9.

Pollow, K., Lubbert, H., Boquoi, E. & Pollow, B. (1975). Progesterone metabolism in normal human endometrium during the menstrual cycle and in endometrial carcinoma. J Clin Endocrinol Metab. 41**:** 729-37.

Ponka, P. (1999). Cell biology of heme. Am J Med Sci. 318**:** 241-56.

Purohit, A., Fusi, L., Brosens, J., Woo, L.W., Potter, B.V. & Reed, M.J. (2008). Inhibition of steroid sulphatase activity in endometriotic implants by 667 COUMATE: a potential new therapy. Hum Reprod. 23**:** 290-7.

Quinlan, G.J., Lagan, A.L. & Evans, T.W. (2008). Haem oxygenase: a model for therapeutic intervention. Intensive Care Med. 34**:** 595-7.

Rahman, I. & Adcock, I.M. (2006). Oxidative stress and redox regulation of lung inflammation in COPD. Eur Respir J. 28**:** 219-42.

Raman, M., Chen, W. & Cobb, M.H. (2007). Differential regulation and properties of MAPKs. Oncogene. 26**:** 3100-12.

Ramey, J.W. & Archer, D.F. (1993). Peritoneal fluid: its relevance to the development of endometriosis. Fertil Steril. 60**:** 1-14.

Razzi, S., Fava, A., Sartini, A., De Simone, S., Cobellis, L. & Petraglia, F. (2004). Treatment of severe recurrent endometriosis with an aromatase inhibitor in a young ovariectomised woman. Bjog. 111**:** 182-4.

Rizner, T.L. (2009). Estrogen metabolism and action in endometriosis. Mol Cell Endocrinol. 307**:** 8-18.

Rizner, T.L., Smuc, T., Rupreht, R., Sinkovec, J. & Penning, T.M. (2006). AKR1C1 and AKR1C3 may determine progesterone and estrogen ratios in endometrial cancer. Mol Cell Endocrinol. 248**:** 126-35.

Rogers, P.A., D'Hooghe, T.M., Fazleabas, A., Gargett, C.E., Giudice, L.C., Montgomery, G.W., Rombauts, L., Salamonsen, L.A. & Zondervan, K.T. (2009). Priorities for endometriosis research: recommendations from an international consensus workshop. Reprod Sci. 16**:** 335-46.

Rohrdanz, E., Ohler, S., Tran-Thi, Q.H. & Kahl, R. (2002). The phytoestrogen daidzein affects the antioxidant enzyme system of rat hepatoma H4IIE cells. J Nutr. 132**:** 370-5.

Roman-Blas, J.A., Castaneda, S., Largo, R. & Herrero-Beaumont, G. (2009). Osteoarthritis associated with estrogen deficiency. Arthritis Res Ther. 11**:** 241.

Rosenblat, M., Coleman, R. & Aviram, M. (2002). Increased macrophage glutathione content reduces cell-mediated oxidation of LDL and atherosclerosis in apolipoprotein E-deficient mice. Atherosclerosis. 163**:** 17-28.

Roux, P.P. & Blenis, J. (2004). ERK and p38 MAPK-activated protein kinases: a family of protein kinases with diverse biological functions. Microbiol Mol Biol Rev. 68**:** 320-44.

Roy, J., Pallepati, P., Bettaieb, A., Tanel, A. & Averill-Bates, D.A. (2009). Acrolein induces a cellular stress response and triggers mitochondrial apoptosis in A549 cells. Chem Biol Interact. 181**:** 154-67.

Ryan, I.P. & Taylor, R.N. (1997). Endometriosis and infertility: new concepts. Obstet Gynecol Surv. 52**:** 365-71.

Ryter, S.W., Alam, J. & Choi, A.M. (2006). Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications. Physiol Rev. 86**:** 583-650.

Sadler, E.M., Reddy, V.R. & Piper, W.N. (1986). Increased rat testicular heme oxygenase activity associated with depressed microsomal heme and cytochrome P-450 levels after repeated administration of human chorionic gonadotropin. Arch Biochem Biophys. 249**:** 382-7.

Saito, Y., Kondo, H. & Hojo, Y. (2011). Granzyme B as a novel factor involved in cardiovascular diseases. J Cardiol. 57**:** 141-7.

Santanam, N., Murphy, A.A. & Parthasarathy, S. (2002). Macrophages, oxidation, and endometriosis. Ann N Y Acad Sci. 955**:** 183-98; discussion 19-200, 396-406.

Sato, N., Ueno, T., Kubo, K., Suzuki, T., Tsukimura, N., Att, W., Yamada, M., Hori, N., Maeda, H. & Ogawa, T. (2009). N-Acetyl cysteine (NAC) inhibits proliferation, collagen gene transcription, and redox stress in rat palatal mucosal cells. Dent Mater. 25**:** 1532-40.

Satoh, K., Nonaka, R., Ishikawa, F., Ogata, A. & Nagai, F. (2008). In vitro screening assay for detecting aromatase activity using rat ovarian microsomes and estrone ELISA. Biol Pharm Bull. 31**:** 357-62.

Schipper, H.M. (2011). Heme oxygenase-1 in Alzheimer disease: a tribute to Moussa Youdim. J Neural Transm. 118**:** 381-7.

Scholz, H. (2003). Prostaglandins. Am J Physiol Regul Integr Comp Physiol. 285**:** R512-4.

Schweppe, K.W. (2001). Current place of progestins in the treatment of endometriosis-related complaints. Gynecol Endocrinol. 15 Suppl 6**:** 22-8.

Seeber, B.E., Czech, T., Buchner, H., Barnhart, K.T., Seger, C., Daxenbichler, G., Wildt, L. & Dieplinger, H. (2010). The vitamin E-binding protein afamin is altered significantly in the peritoneal fluid of women with endometriosis. Fertil Steril. 94**:** 2923-6.

Sen, C.K. (1998). Redox signaling and the emerging therapeutic potential of thiol antioxidants. Biochem Pharmacol. 55**:** 1747-58.

Sepulcri Rde, P. & do Amaral, V.F. (2009). Depressive symptoms, anxiety, and quality of life in women with pelvic endometriosis. Eur J Obstet Gynecol Reprod Biol. 142**:** 53-6.

Seval, Y., Cakmak, H., Kayisli, U.A. & Arici, A. (2006). Estrogen-mediated regulation of p38 mitogen-activated protein kinase in human endometrium. J Clin Endocrinol Metab. 91**:** 2349-57.

Seyfried, J., Soldner, F., Kunz, W.S., Schulz, J.B., Klockgether, T., Kovar, K.A. & Wullner, U. (2000). Effect of 1-methyl-4-phenylpyridinium on glutathione in rat pheochromocytoma PC 12 cells. Neurochem Int. 36**:** 489-97.

Shanti, A., Santanam, N., Morales, A.J., Parthasarathy, S. & Murphy, A.A. (1999). Autoantibodies to markers of oxidative stress are elevated in women with endometriosis. Fertil Steril. 71**:** 1115-8.

Sharpe-Timms, K.L. & Young, S.L. (2004). Understanding endometriosis is the key to successful therapeutic management. Fertil Steril. 81**:** 1201-3.

Shimizu, Y., Takeuchi, T., Mita, S., Mizuguchi, K., Kiyono, T., Inoue, M. & Kyo, S. (2009). Dienogest, a synthetic progestin, inhibits the proliferation of immortalized human endometrial epithelial cells with suppression of cyclin D1 gene expression. Mol Hum Reprod. 15**:** 693-701.

Shurtz-Swirski, R., Sela, S., Herskovits, A.T., Shasha, S.M., Shapiro, G., Nasser, L. & Kristal, B. (2001). Involvement of peripheral polymorphonuclear leukocytes in oxidative stress and inflammation in type 2 diabetic patients. Diabetes Care. 24**:** 104-10.

Signorile, P.G. & Baldi, A. (2010). Endometriosis: new concepts in the pathogenesis. Int J Biochem Cell Biol. 42**:** 778-80.

Simoncini, T., Rabkin, E. & Liao, J.K. (2003). Molecular basis of cell membrane estrogen receptor interaction with phosphatidylinositol 3-kinase in endothelial cells. Arterioscler Thromb Vasc Biol. 23**:** 198-203.

Slebos, D.J., Ryter, S.W. & Choi, A.M. (2003). Heme oxygenase-1 and carbon monoxide in pulmonary medicine. Respir Res. 4**:** 7.

Smuc, T., Hevir, N., Ribic-Pucelj, M., Husen, B., Thole, H. & Rizner, T.L. (2009). Disturbed estrogen and progesterone action in ovarian endometriosis. Mol Cell Endocrinol. 301**:** 59-64.

Smuc, T. & Rizner, T.L. (2009). Expression of 17beta-hydroxysteroid dehydrogenases and other estrogen-metabolizing enzymes in different cancer cell lines. Chem Biol Interact. 178**:** 228-33.

Solaini, G., Sgarbi, G. & Baracca, A. (2011). Oxidative phosphorylation in cancer cells. Biochim Biophys Acta.

Stadtman, E.R. (2004). Role of oxidant species in aging. Curr Med Chem. 11**:** 1105-12.

Stefansson, H., Geirsson, R.T., Steinthorsdottir, V., Jonsson, H., Manolescu, A., Kong, A., Ingadottir, G., Gulcher, J. & Stefansson, K. (2002). Genetic factors contribute to the risk of developing endometriosis. Hum Reprod. 17**:** 555-9.

Stevens, J.F. & Maier, C.S. (2008). Acrolein: sources, metabolism, and biomolecular interactions relevant to human health and disease. Mol Nutr Food Res. 52**:** 7-25.

Stocker, R., Yamamoto, Y., McDonagh, A.F., Glazer, A.N. & Ames, B.N. (1987). Bilirubin is an antioxidant of possible physiological importance. Science. 235**:** 1043-6.

Strniskova, M., Barancik, M. & Ravingerova, T. (2002). Mitogen-activated protein kinases and their role in regulation of cellular processes. Gen Physiol Biophys. 21**:** 231-55.

Sueldo, C.E., Lambert, H., Steinleitner, A., Rathwick, G. & Swanson, J. (1987). The effect of peritoneal fluid from patients with endometriosis on murine sperm-oocyte interaction. Fertil Steril. 48**:** 697-9.

Sugino, N., Shimamura, K., Takiguchi, S., Tamura, H., Ono, M., Nakata, M., Nakamura, Y., Ogino, K., Uda, T. & Kato, H. (1996). Changes in activity of superoxide dismutase in the human endometrium throughout the menstrual cycle and in early pregnancy. Hum Reprod. 11**:** 1073-8.

Szczepanska, M., Kozlik, J., Skrzypczak, J. & Mikolajczyk, M. (2003). Oxidative stress may be a piece in the endometriosis puzzle. Fertil Steril. 79**:** 1288-93.

Tamura, M., Sebastian, S., Gurates, B., Yang, S., Fang, Z. & Bulun, S.E. (2002a). Vascular endothelial growth factor up-regulates cyclooxygenase-2 expression in human endothelial cells. J Clin Endocrinol Metab. 87**:** 3504-7.

Tamura, M., Sebastian, S., Yang, S., Gurates, B., Fang, Z. & Bulun, S.E. (2002b). Interleukin-1beta elevates cyclooxygenase-2 protein level and enzyme activity via increasing its mRNA stability in human endometrial stromal cells: an effect mediated by extracellularly regulated kinases 1 and 2. J Clin Endocrinol Metab. 87**:** 3263-73.

Thannickal, V.J. & Fanburg, B.L. (2000). Reactive oxygen species in cell signaling. Am J Physiol Lung Cell Mol Physiol. 279**:** L1005-28.

Uchida, K., Kanematsu, M., Sakai, K., Matsuda, T., Hattori, N., Mizuno, Y., Suzuki, D., Miyata, T., Noguchi, N., Niki, E. & Osawa, T. (1998). Protein-bound acrolein: potential markers for oxidative stress. Proc Natl Acad Sci U S A. 95**:** 4882-7.

Ueki, M. (1991). Histologic study of endometriosis and examination of lymphatic drainage in and from the uterus. Am J Obstet Gynecol. 165**:** 201-9.

Ulusu, N.N. & Tandogan, B. (2007). Purification and kinetic properties of glutathione reductase from bovine liver. Mol Cell Biochem. 303**:** 45-51.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T., Mazur, M. & Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol. 39**:** 44-84.

Valko, M., Rhodes, C.J., Moncol, J., Izakovic, M. & Mazur, M. (2006). Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chem Biol Interact. 160**:** 1-40.

Valle, R.F. (2002). Endometriosis: current concepts and therapy. Int J Gynaecol Obstet. 78**:** 107-19.

Valle, R.F. & Sciarra, J.J. (2003). Endometriosis: treatment strategies. Ann N Y Acad Sci. 997**:** 229-39.

Van Langendonckt, A., Casanas-Roux, F., Dolmans, M.M. & Donnez, J. (2002a). Potential involvement of hemoglobin and heme in the pathogenesis of peritoneal endometriosis. Fertil Steril. 77**:** 561-70.

Van Langendonckt, A., Casanas-Roux, F. & Donnez, J. (2002b). Iron overload in the peritoneal cavity of women with pelvic endometriosis. Fertil Steril. 78**:** 712-8.

Van Langendonckt, A., Casanas-Roux, F. & Donnez, J. (2002c). Oxidative stress and peritoneal endometriosis. Fertil Steril. 77**:** 861-70.

Velarde, M.C., Aghajanova, L., Nezhat, C.R. & Giudice, L.C. (2009). Increased mitogen-activated protein kinase kinase/extracellularly regulated kinase activity in human endometrial stromal fibroblasts of women with endometriosis reduces 3',5'-cyclic adenosine 5'-monophosphate inhibition of cyclin D1. Endocrinology. 150**:** 4701-12.

Vercellini, P., Crosignani, P., Somigliana, E., Vigano, P., Frattaruolo, M.P. & Fedele, L. (2011). 'Waiting for Godot': a commonsense approach to the medical treatment of endometriosis. Hum Reprod. 26**:** 3-13.

Verderame, M. & Limatola, E. (2010). Molecular identification of estrogen receptors (ERalpha and ERbeta) and their differential expression during VTG synthesis in the liver of lizard Podarcis sicula. Gen Comp Endocrinol. 168**:** 231-8.

Vigano, P., Parazzini, F., Somigliana, E. & Vercellini, P. (2004). Endometriosis: epidemiology and aetiological factors. Best Pract Res Clin Obstet Gynaecol. 18**:** 177-200.

Vinatier, D., Orazi, G., Cosson, M. & Dufour, P. (2001). Theories of endometriosis. Eur J Obstet Gynecol Reprod Biol. 96**:** 21-34.

Waisberg, M., Joseph, P., Hale, B. & Beyersmann, D. (2003). Molecular and cellular mechanisms of cadmium carcinogenesis. Toxicology. 192**:** 95-117.

Waller, K.G., Lindsay, P., Curtis, P. & Shaw, R.W. (1993). The prevalence of endometriosis in women with infertile partners. Eur J Obstet Gynecol Reprod Biol. 48**:** 135-9.

Walters, M.R. (1985). Steroid hormone receptors and the nucleus. Endocr Rev. 6**:** 512-43.

Wang, J., Sun, P., Bao, Y., Liu, J. & An, L. (2010). Cytotoxicity of single-walled carbon nanotubes on PC12 cells. Toxicol In Vitro. 25**:** 242-50.

Wang, J., Sun, P., Bao, Y., Liu, J. & An, L. (2011). Cytotoxicity of single-walled carbon nanotubes on PC12 cells. Toxicol In Vitro. 25**:** 242-50.

Wang, Y., Ji, H.X., Xing, S.H., Pei, D.S. & Guan, Q.H. (2007). SP600125, a selective JNK inhibitor, protects ischemic renal injury via suppressing the extrinsic pathways of apoptosis. Life Sci. 80**:** 2067-75.

Wang, Y., Sharma, R.K., Falcone, T., Goldberg, J. & Agarwal, A. (1997). Importance of reactive oxygen species in the peritoneal fluid of women with endometriosis or idiopathic infertility. Fertil Steril. 68**:** 826-30.

Weber, K. & Osborn, M. (1969). The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J Biol Chem. 244**:** 4406-12.

Wei, M.C., Zong, W.X., Cheng, E.H., Lindsten, T., Panoutsakopoulou, V., Ross, A.J., Roth, K.A., MacGregor, G.R., Thompson, C.B. & Korsmeyer, S.J. (2001). Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science. 292**:** 727-30.

Weston, C.R. & Davis, R.J. (2007). The JNK signal transduction pathway. Curr Opin Cell Biol. 19**:** 142-9.

Wilhelm, F., Kovacs, K.A., Lengyel, F., Menyhart, C., Vizer, M.G., Vertes, M. & Szabo, I. (2011). Akt/protein B and estradiol receptor alpha expressions in postmenopausal endometrium. Eur J Obstet Gynecol Reprod Biol.

Willcox, J.K., Ash, S.L. & Catignani, G.L. (2004). Antioxidants and prevention of chronic disease. Crit Rev Food Sci Nutr. 44**:** 275-95.

Wong, H.R. & Wispe, J.R. (1997). The stress response and the lung. Am J Physiol. 273**:** L1-9.

Wren, J.D., Wu, Y. & Guo, S.W. (2007). A system-wide analysis of differentially expressed genes in ectopic and eutopic endometrium. Hum Reprod. 22**:** 2093-102.

Wu, M.H., Sun, H.S., Lin, C.C., Hsiao, K.Y., Chuang, P.C., Pan, H.A. & Tsai, S.J. (2002). Distinct mechanisms regulate cyclooxygenase-1 and -2 in peritoneal macrophages of women with and without endometriosis. Mol Hum Reprod. 8**:** 1103-10.

Wu, M.H., Wang, C.A., Lin, C.C., Chen, L.C., Chang, W.C. & Tsai, S.J. (2005). Distinct regulation of cyclooxygenase-2 by interleukin-1beta in normal and endometriotic stromal cells. J Clin Endocrinol Metab. 90**:** 286-95.

Wu, Y. & Guo, S.W. (2006). Inhibition of proliferation of endometrial stromal cells by trichostatin A, RU486, CDB-2914, N-acetylcysteine, and ICI 182780. Gynecol Obstet Invest. 62**:** 193-205.

Wu, Y., Kajdacsy-Balla, A., Strawn, E., Basir, Z., Halverson, G., Jailwala, P., Wang, Y., Wang, X., Ghosh, S. & Guo, S.W. (2006). Transcriptional characterizations of differences between eutopic and ectopic endometrium. Endocrinology. 147**:** 232-46.

Xue, Q., Lin, Z., Cheng, Y.H., Huang, C.C., Marsh, E., Yin, P., Milad, M.P., Confino, E., Reierstad, S., Innes, J. & Bulun, S.E. (2007). Promoter methylation regulates estrogen receptor 2 in human endometrium and endometriosis. Biol Reprod. 77**:** 681-7.

Yoshida, M., Tomitori, H., Machi, Y., Hagihara, M., Higashi, K., Goda, H., Ohya, T., Niitsu, M., Kashiwagi, K. & Igarashi, K. (2009). Acrolein toxicity: Comparison with reactive oxygen species. Biochem Biophys Res Commun. 378**:** 313-8.

Yoshino, O., Osuga, Y., Hirota, Y., Koga, K., Hirata, T., Harada, M., Morimoto, C., Yano, T., Nishii, O., Tsutsumi, O. & Taketani, Y. (2004). Possible pathophysiological roles of mitogen-activated protein kinases (MAPKs) in endometriosis. Am J Reprod Immunol. 52**:** 306-11.

Young, B.P.D.W.s.f.h. & Wheater, P.R.F.h. (2006). *Wheater's functional histology : a text and colour atlas,* [Edinburgh], Churchill Livingstone Elsevier.

Yu, F.Y., Wu, T.S., Chen, T.W. & Liu, B.H. (2011). Aristolochic acid I induced oxidative DNA damage associated with glutathione depletion and ERK1/2 activation in human cells. Toxicol In Vitro. 25**:** 810-6.

Zafarullah, M., Li, W.Q., Sylvester, J. & Ahmad, M. (2003). Molecular mechanisms of N-acetylcysteine actions. Cell Mol Life Sci. 60**:** 6-20.

Zeitoun, K., Takayama, K., Michael, M.D. & Bulun, S.E. (1999). Stimulation of aromatase P450 promoter (II) activity in endometriosis and its inhibition in endometrium are regulated by competitive binding of steroidogenic factor-1 and chicken ovalbumin upstream promoter transcription factor to the same cis-acting element. Mol Endocrinol. 13**:** 239-53.

Zeitoun, K., Takayama, K., Sasano, H., Suzuki, T., Moghrabi, N., Andersson, S., Johns, A., Meng, L., Putman, M., Carr, B. & Bulun, S.E. (1998). Deficient 17beta-hydroxysteroid dehydrogenase type 2 expression in endometriosis: failure to metabolize 17beta-estradiol. J Clin Endocrinol Metab. 83**:** 4474-80.

Zeitoun, K.M. & Bulun, S.E. (1999). Aromatase: a key molecule in the pathophysiology of endometriosis and a therapeutic target. Fertil Steril. 72**:** 961-9.

Zeitvogel, A., Baumann, R. & Starzinski-Powitz, A. (2001). Identification of an invasive, N-cadherin-expressing epithelial cell type in endometriosis using a new cell culture model. Am J Pathol. 159**:** 1839-52.

Zeller, J.M., Henig, I., Radwanska, E. & Dmowski, W.P. (1987). Enhancement of human monocyte and peritoneal macrophage chemiluminescence activities in women with endometriosis. Am J Reprod Immunol Microbiol. 13**:** 78-82.

Zhang, H., Zhao, X., Liu, S., Li, J., Wen, Z. & Li, M. (2010). 17betaE2 promotes cell proliferation in endometriosis by decreasing PTEN via NFkappaB-dependent pathway. Mol Cell Endocrinol. 317**:** 31-43.

Zhang, W. & Liu, H.T. (2002). MAPK signal pathways in the regulation of cell proliferation in mammalian cells. Cell Res. 12**:** 9-18.

RESEARCH OUTPUTS

Noordin L, Elizabeth M Ellis. Heme Oxygenase-1 as a potential biomarker in the pathogenesis of endometriosis. *Proceedings for The Physiological Society Meeting, Dublin* (July 2009).

Noordin L, Elizabeth M Ellis. Estradiol enhanced oxidative stress induced endometriotic cell proliferation.

- *Proceedings for Endometriosis 2010 from bench to patient (Biology and clinical investigations), Milan, Italy* (March 2010).

- *University of Strathclyde Research Day* (June 2010)
  (winner for best poster for Science Category).

Noordin L, Elizabeth M Ellis. N-Acetylcysteine inhibits proliferation in endometriotic cells.

- *Proceedings for The Physiological Society Meeting, Manchester* (July 2010). (*winner for best poster in the Metabolism & Endocrinology Theme*)

- *Strathclyde Institute of Pharmacy and Biomedical Sciences (SIPBS) Annual Research Symposium, University of Strathclyde* (September 2010).

Noordin L, Rizner, T.L, Elizabeth M Ellis. Estradiol and oxidative stress induced the expression of aromatase, steroid receptors and steroid pre-receptor regulators genes in endometriotic cells. *Proceedings for The 13th World Congress on Controversies in Obstetrics, Gynecology & Infertility (COGI), Berlin, Germany* (November 2010).

Noordin L, Rizner, T.L, Elizabeth M Ellis. Induction of aromatase expression and activity and steroid receptors expression in endometriotic cells: effects of oxidative stress and estradiol. *Proceedings for The Physiological Society Meeting, Durrham* (December 2010).

Noordin L, Elizabeth M Ellis. The 'Positive Feedback Loop' of estradiol production in endometriotic epithelial cells. *Proceedings for The 19th International Pelvic Pain Society Annual Scientific Meeting, Turkey* (May 2011).