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Evaluation of antioxidants encapsulated nanoparticles in animal models

By

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

Signed: *Date:*

Dedication

I dedicate this research work to my parents, sister and brother for their love and support.

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Contents

ABSTRACT

Antioxidants are known to be effective in diseases where oxidative stress is implicated. Ellagic acid (EA), coenzyme Q_{10} (CoQ_{10}) and curcumin are potent antioxidants *in vitro* but suffer from low oral bioavailability. Therefore, we formulated antioxidants encapsulated biodegradable nanoparticles to improve their *in vivo* performance. Nano-co-encapsulated antioxidant particles (NanoCAPs) of EA and CoQ_{10} combination, CoQ_{10} nanoparticles and curcumin nanoparticles were prepared using emulsion-diffusion-evaporation method. NanoCAPs were evaluated in high fat diet fed rats and found to be effective in normalising the hypercholesterolaemia, hypertriglyceridaemia and hyperglycaemia although the mechanisms of action need to be further studied. NanoCAPs showed sustained action on the cholesterol levels for prolonged durations compared to the suspension formulation. Curcumin nanoparticles lowered the interleukin-6, tumour necrosis factor-α and C-reactive protein levels whereas CoQ_{10} nanoparticles reduced only C-reactive protein levels in the plasma of diabetic rats. CoQ_{10} and curcumin nanoparticles normalised the lipid levels in diabetic rats. In chronic hypoxic rats, CoQ_{10} and curcumin have shown no significant effects on haematocrit, right ventricular hypertrophy and vascular remodelling in the lung. Histological examination showed the presence of curcumin nanoparticles in lungs and tissue distribution studies revealed altered distribution under hypoxia.

β-galactosidase encapsulation into nanoparticles was optimised and found to be capable in retaining ~48% of the initially loaded active enzyme. Histological examination of the orally administered β-gal nanoparticles in the rat intestinal wall suggested that the nanoparticles are absorbed intact.

LIST OF ABBREVIATIONS

5-HT	-	5-hydroxy tryptamine or serotonin
5-HTT	-	5-hydroxy tryptamin transporter
β-gal	-	β-galactosidase
ABTS	-	2,20-azinobis(3-ethylbenzthiazoline-6-sulphonic acid)
Ach	-	Acetyl choline
AFM	-	Atomic force microscope
AGEs	-	Advanced glycation end products
ANOVA	-	Analysis of variance
ATP	-	Adenosine triphosphate
BHA	-	Butylated hydroxyanisole
BHT	-	Butylated hydroxytoluene
cGMP	-	Cyclic guanosine monophosphate
CHD	-	Coronary heart diseases
CHL	-	Chang human liver
CoQ_{10}	-	Coenzyme Q_{10} or ubiquinone
$CoQ_{10}H_2$	-	Reduced CoQ_{10} or ubiquinol
C_{max}	-	Plasma peak concentration
CRP	-	C-reactive protein
CVD	-	Cardiovascular diseases
DAB	-	3,3-diaminobenzidinetetra hydrochloride
DAG	-	Diacyl glycerol
DCFH-DA	-	Dichlorofluorescein diacetate
DNA	-	Deoxyribonucleic acid

DMAB	-	Didodecyl dimethyl ammonium bromide
DMPD	-	N,N-dimethyl-p-phenylenediamine dihydrochloride
DPPH	-	2,2-diphenyl-1-picrylhydrazyl
EA	-	Ellagic acid
ecSOD	-	Extra cellular superoxide dismutase
ED_{50}	-	Effective dose
EDE	-	Emulsion diffusion evaporation
EDTA	-	Ethylenediaminetetra acetic acid
EE	-	Entrapment efficiency
eNOS	-	Endothelial nitric oxide synthase
ET-1	-	Endothelin-1
ET_A	-	Endothelin receptor A
ET_B	-	Endothelin receptor B
FDA	-	Food and drugs administration
FRAP	-	Ferric reducing antioxidant capacity
FSP	-	Freeze dried strawberry powder
GALT	-	Gut associated lymphoid tissue
GI	-	Gastro intestinal
GK	-	Goto-Kakizaki
GSH	-	Glutathione
GPx	-	Glutathione peroxidase
H_2O_2	-	Hydrogen peroxide
H&E	-	Haematoxylin and eosin

HbA1C	-	Glycated haemoglobin
HBE	-	Human bronchial epithelium
HDL-C	-	High density lipoprotein cholesterol
HFD	-	High fat diet
HIV	-	Human immunodeficiency virus
HMG-CoA	-	3-hydroxy-3-methyl-glutaryl-CoA reductase
HPV	-	Hypoxic pulmonary vasoconstriction
hs-CRP	-	high sensitivity C-reactive protein
HUVEC	-	Human umbilical endothelial cells
IDL	-	Intermediate density lipoprotein
IL-6	-	Interleukin-6
IPAH	-	Idiopathic pulmonary arterial hypertension
KHB	-	Krebs' Henseleit buffer
LDL	-	Low density lipoprotein
LDL-C	-	Low density lipoprotein cholesterol
mcEA	-	Microspheres of ellagic acid
MCT	-	Monocrotaline
MDA	-	Malondialdehyde
MHTG	-	Massive hypertriglyceridaemia
mPAP	-	Mean pulmonary arterial pressure
NAC	-	N-acetyl cysteine
NADH	-	Nicotinamide adenine dinucleotide
NADPH	-	Nicotinamide adenine dinucleotide phosphate

NanoCAPs	-	Nano-co-encapsulated antioxidant particles
NCEP	-	National Cholesterol Education Program
NF-κB	-	Nuclear factor- κB
NGS	-	Normal goat serum
NICE	-	National institute for health and clinical excellence
NOS	-	Nitric oxide synthase
NOX	-	NADPH oxidase
NPD	-	Normal pellet diet
OK	-	Opossum kidney
ONPG	-	Ortho-nitrophenyl-β-galactoside
ORAC	-	Oxygen radical absorption capacity
PAH	-	Pulmonary arterial hypertension
PASMC	-	Pulmonary arterial smooth muscle cell
PBS	-	Phosphate buffered saline
PCOOH	-	Phosphatidylcholine hydroperoxide
PDE5	-	Phosphodiesterase type 5
PE	-	Phenylephrine
PEG	-	Poly ethylene glycol
PGI2	-	Prostacyclin
PH	-	Pulmonary hypertension
PKC	-	Protein kinase-C
PLGA	-	Poly (lactic-co-glycolic) acid
PPHN	-	Persistent pulmonary hypertension

PRA	-	Resistance pulmonary arteries
PVA	-	Poly vinyl alcohol
PVA-CS	-	Poly vinyl alcohol-chitosan blend
RCT	-	Reverse cholesterol transport
ROS	-	Reactive oxygen species
SEM	-	Standard error of the mean
SOD	-	Superoxide dismutase
STZ	-	Streptozotocin
T1D	-	Type 1 Diabetes mellitus
T2D	-	Type 2 Diabetes mellitus
TBARS	-	Thiobarbituric acid reactive substances
TC	-	Total cholesterol
TEM	-	Transmission electron microscope
TG	-	Triglycerides
TNF-α	-	Tumour necrosis factor-α
VEGF	-	Vascular endothelial growth factor
VLDL	-	Very low density lipoprotein
VLDL-C	-	Very low density lipoprotein-cholesterol
WHO	-	World health organisation
X-gal	-	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
XO	-	Xanthine oxidase

1. GENERAL INTRODUCTION

The role of reactive oxygen species (ROS) in pathogenesis and progression of many disease conditions is known [Touyz *et al.* 2011]. ROS are highly reactive molecules. Under normal conditions ROS are transformed into less harmful products by the enzymes super oxide dismutase (SOD), catalase and glutathione peroxidise (GPx). ROS are the side products of respiration in the mitochondria. ROS generation is initiated with the super oxide radical which under normal conditions is dismutated to hydrogen peroxide (H_2O_2) by the enzyme SOD. However, H_2O_2 formed in this process reacts with various cell components and forms ROS. H_2O_2 is broken down by the catalase enzyme to give water and oxygen. In normal conditions, ROS help inmetabolic signalling, but when produced in excess under certain conditions like hypoxia or reoxygenation can also directly damage the cells or their components. Thus it is essential to have the balance between ROS and the antioxidant levels in the cells, otherwise this destructive function of the electron transport chain represents the main problem in many systemic diseases [Selivanov *et al.* 2011].

As the understanding is increasing on the free radical associated damage the demand for antioxidants from various sources is also increasing. *In vitro* extrapolation and preclinical experiments present the benefits of antioxidants. However, at clinical set up antioxidants fail to show expected results because of their poor biopharmaceutical properties and instability [Sheikh-Ali *et al.* 2009, Marinangeli and Jones 2010]. This paradoxical situation is hindering the pharmaceutical development of antioxidants, which can be equally effective as other classes of pharmaceutical agents in many diseases. Due to the poor performance in clinical trials, presently, antioxidants are only regarded as supplementary therapy [Halliwell 2000].

Efforts from researchers are underway to overcome this paradoxical situation by banking upon novel delivery systems. Novel delivery systems can improve the deliverability of antioxidants by improving the poor biopharmaceutical properties and stability [Ratnam *et al.* 2006, Belcaro *et al.* 2010, Saraf 2010]. Using the novel delivery systems of antioxidants, instead of using the dietary supplements or the conventional formulations in clinical trials could offer potential benefits.

Ellagic acid (EA), coenzyme Q_{10} (CoQ_{10}) and curcumin were selected for the present research program, because of their potential activities as antioxidants and anti-inflammatory agents. In the following section, the efficacy, drawbacks and the pharmaceutical approaches to overcome the drawbacks of the selected molecules will be discussed. Later on the disease conditions hyperlipidaemia, diabetes and pulmonary hypertension will be discussed in which the developed nanoparticles are to be tested. Finally, the nanoparticulate approach and the ability of the nanoparticles to increase the bioavailability and their uptake mechanisms will be discussed in the general introduction.

1. 1. Overview of antioxidants

1.1.1. Ellagic acid

Ellagic acid (EA) is a natural polyphenolic compound that occurs largely as ellagitannins. It is found in different fruits and nuts, like pomegranate, red raspberry, strawberry, blueberry and walnuts [Basu *et al.* 2009, Landete 2011]. EA is a dimeric derivative of gallic acid and is generated by the hydrolysis of ellagitannins [Girish and Pradhan 2008]. It is known to possess a variety of pharmacological activities such as antioxidant, anti-inflammatory, antimutagenic, antitumoral, antihyperglycaemic and anti-infective activity [Landete 2011].

EA has shown a strong *in vitro* antioxidant activity in 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant capacity (FRAP), oxygen radical absorption capacity (ORAC) and 2,20-azinobis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) assays [Hayes *et al.* 2011]. Antioxidant activity usually depends on the numbers and positions of the hydroxyl groups in relation to the carboxyl functional group [Rice-Evans *et al.* 1996]. The potent radical scavenging capacity of EA is not surprising as this compound has four phenolic OH groups with a fused benzofuran structure [Table 1.1].

In cell culture studies EA is a proven antioxidant. The dichlorofluorescein diacetate (DCFH-DA) cytofluorimetric test ascertains the strong scavenging action exerted by EA in Chinese hamster ovary cells [Festa *et al.* 2001]. There was an increase in DCF fluorescence in Chang human liver (CHL) cells treated with 60 µM vitamin K3, and the treatment of CHL cells with 2 mM glutathione (GSH) or 10 µM EA lowered DCF fluorescence to normal levels [Hwang *et al.* 2010]. From this study it was revealed that EA is more potent than endogenous antioxidant GSH. EA was also potent than N-acetyl cystein (NAC) in alleviating nicotine induced oxidative stress. 100 µM EA significantly normalised 3 mM nicotine induced increase in thiobarbituric acid reactive substances (TBARS) and decrease in reduced glutathione (GSH), whereas 1mM NAC was required to show similar effects in rat peripheral blood lymphocytes [Sudheer *et al.* 2007].

Various experimental and clinical studies have shown that atleast two fold increase in the oxidative stress is observed in myocardial infarction [Rathore *et al.* 1998]. Antioxidants can interrupt the free radicals mediated myocardial injury [Rajadurai and Prince 2006, Srivastava *et al.* 2007]. Oral pretreatment with EA (7.5 & 15 mg/kg) for ten days, significantly reduced the levels of TBARS, lipid hydroperoxides, necrosis and invasion of neutrophils

in the heart tissue of isoproterenol treated rats. Pre-treatment of EA also increased the activities of SOD, catalase, GPx, glutathione-s-transferase and the levels of reduced GSH in the heart tissue homogenate [Kannan and Quine 2011].

Lipid peroxidation in the form of malondialdehyde (MDA) and hydroxynonenal in the plasma was found to be significantly reduced with freeze dried strawberry powder treatment for four weeks in female subjects with metabolic syndrome. Oxidized-LDL showed a decreasing trend at 4th week. Strawberry powder is rich in EA and a significant number of subjects (13/16) showed an increase in plasma EA after four weeks treatment [Basu *et al.* 2009].

Besides being an antioxidant, EA also acts on drug-metabolising enzymes and prevents the formation of toxic metabolites [Hayeshi *et al.* 2007]. EA suppresses nuclear factor-κB (NF-κB) activation in vascular endothelial cells in response to biochemical or biomechanical stimuli, which is associated with vascular pathologies such as atherosclerosis [Shubert *et al.* 2002]. Antiangiogenic activity of EA can be related to the chelation of divalent cations and inactivation of metalloproteinases [Losso *et al.* 2004]. EA inhibits carcinogen bioactivation, binding of carcinogens to DNA and inhibits growth promotion in cancer cells [Whitley *et al.* 2003]. Apart from these effects, the aldose reductase inhibitor activity was also reported for this molecule [Ueda *et al.* 2004]. EA was shown to possess antihyperlipidaemic activities in mice [Lei *et al.* 2007] and in rabbits [Yu *et al.* 2005]. EA was also useful in amelioration of drug associated adverse effects caused due to free radical production. Ciclosporine induces kidney damage through free radical production and it was shown to be significantly lowered by EA treatment in rats [Sonaje *et al.* 2007].

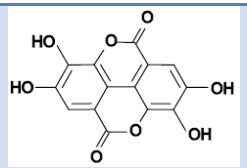
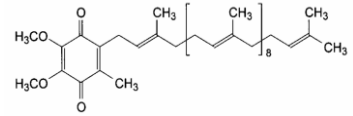
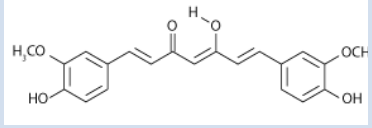
The poor solubility of EA in aqueous medium, which is below 10 μg/ml, poses challenge in the delivery of this molecule [Table 1.1]. The low concentration of free EA in plasma has been attributed to its poor solubility resulting in poor absorption from gastrointestinal (GI) tract and may also be because of its excessive metabolic transformation and degradation prior to absorption [Lei *et al.* 2003]. Irreversible binding of EA to cellular DNA and proteins in the enterocytes may also account for its limited transcellular absorption from the gut wall [Whitley *et al.* 2003]. Low absorption may also be due to ionization of EA at physiological pH and formation of insoluble complexes of EA with Mg^{+2} and Ca^{+2} ions [Larossa *et al.* 2010]. EA is thought to be metabolised by the intestinal microbes after oral administration. The germ free rat faeces showed the presence of unchanged EA where as normal rats doesn't show any presence of free EA. 10% of the orally administered dose was excreted as 3,8-dihydroxy-6H-dibenzo[b,d]pyran-6-one in urine and faeces and two conjugates of the metabolite were detected in the bile [Doyle and Griffiths 1980]. The plasma concentration of EA declined with α (0.8 h) and β (5.0 h) half lives in rats [Lei *et al.* 2003].

To overcome the limitation of poor bioavailability some of the studies have employed novel delivery strategies for EA. A novel dietary formulation, EA-phospholipid complex was developed to investigate the effect on carbon tetrachloride induced liver damage in rats. The antioxidant activity of the orally administered complex (equivalent of 25 and 50 mg/kg body weight of free EA) and free EA (25 and 50 mg/kg of body weight) was evaluated by measuring various enzymes in oxidative stress condition. The phospholipid complex significantly protected the liver by restoring the activity of SOD, catalase, liver GSH, and by reducing the TBARS. The serum concentration of EA obtained from the complex (equivalent to 80 mg/kg of

EA) was higher (C_{max} = 0.54 µg/mL) than that of pure EA (80 mg/kg) (C_{max} = 0.21 µg/mL) suggesting improvement of bioavailability by the complex [Murugan *et al.* 2009]. The severity of dextran sulfate sodium induced colitis in rats was reduced in a dose-dependent manner with microspheres of EA (mcEA). A significant effect was observed at 10 mg/kg, the ED_{50} being 2.3 mg/kg. Although EA alone without using microspheres was also effective in reducing the severity of the colitis, this effect was much less potent as compared with that of mcEA. The ED_{50} was about 15 times higher than that of mcEA for EA [Ogawa *et al.* 2002].

Nanoparticulate approach was adapted for the delivery of EA in our laboratory. A novel method was developed for the preparation of EA nanoparticles using a co-solvent owing to its poor solubility in routinely used solvents for the nanoparticles preparation [Bala *et al.* 2005, Sonaje *et al.* 2007]. *In situ* intestinal permeability studies with EA encapsulated nanoparticles using polyvinyl alcohol (PVA), polyvinyl alcohol-chitosan (PVA-CS) blend and didodecyl dimethyl ammonium bromide (DMAB) as stabilisers showed 75%, 73% and 87% permeation respectively. Simple EA showed only 66% permeation across the intestinal wall. EA nanoparticles showed sustained effect on the scavenging of ROS in yeast cells suggesting a slow release of EA from the nanoparticles [Bala *et al.* 2006b].

Table 1.1. Pharmaceutical aspects of the antioxidants

Antioxidant	Structure	Biopharmaceutical hurdle	Some selected novel delivery approaches
Ellagic acid		Poor solubility (Press and Hardcastle 1969), Poor bioavailability (Lei *et al.* 2003)	Phospholipid complex (Mugrugan *et al* 2009), Microspheres (Ogawa *et al.* 2002), Polymeric nanoparticles (Bala *et al.* 2006a)
Coenzyme Q_{10}		Poor solubility (Bank *et al.* 2011), Poor bioavailability (Bhagavan and Chopra 2006), Photo instability (Bhagavan and Chopra 2006)	Emulsions (Ozawa *et al.* 1986), Dispersions (Westesan and Siekmann 2001), Self-emulsifying drug delivery systems (Kommuru *et al.* 2001), Solid lipid nanoparticles (Wissing *et al.* 2004), Microspheres (Bule *et al.* 2010), Polymeric nanoparticles (Ankola *et al.* 2007)
Curcumin		Poor solubility (Wang *et al.* 1997), Poor bioavailability (Sharma *et al.* 2007), Instability (Wang et al 1997)	Use of bioavailability enhancers (Munjal et al. 2011), Polymeric nanoparticles (Shaikh *et al.* 2007)

1.1.2. Coenzyme Q_{10} (CoQ_{10})

Coenzyme Q_{10} (also known as ubiquinone, ubidecarenone, coenzyme Q and CoQ_{10}) is a benzoquinone, where Q refers to the quinone chemical group and 10 refers to the number of isoprenyl chemical subunits. CoQ_{10} is an oil-soluble vitamin-like substance which is present in most eukaryotic cells, primarily in the mitochondria [Bank *et al.* 2011]. It is an important component of the electron transport chain and participates in aerobic cellular respiration [Ernster *et al.* 1995]. Organs with the highest energy requirements such as the heart, and the liver have the highest CoQ_{10} concentrations [Okamoto *et al.* 1989].

CoQ_{10} does not show any *in vitro* scavenging activity in DPPH assay [Choi *et al.* 2009]. The reduced form of CoQ_{10}, ubiquinol ($CoQ_{10}H_2$), possesses antioxidative properties instead of CoQ_{10}. Thus it is necessary for CoQ_{10} to be converted to $CoQ_{10}H_2$ to show any antioxidant activity. $CoQ_{10}H_2$ was as effective as α-tocopherol in preventing peroxidative damage to multilamellar phosphatidylcholine liposomes. $CoQ_{10}H_2$ spared α-tocopherol when both antioxidants are present in the same liposomal membranes [Frei *et al.* 1990]. A recent study conducted using pulse radiolysis suggests that CoQ_{10} can react with the superoxide radical which provides insights into the antioxidative properties of CoQ_{10}. The scavenging reaction of CoQ_{10} with superoxide generates ubiquinone radical anions which can protonate to form ubisemiquinone radical which is then transformed into $CoQ_{10}H_2$. The antioxidant effects of endogenous and exogenous CoQ_{10} may be due to direct reaction of CoQ_{10} with superoxide apart from the scavenging effect of carbon and oxygen-centered radicals and the recycling of α-tocopherol by $CoQ_{10}H_2$ [Maroz *et al.* 2009].

The antioxidant activity of CoQ_{10} in intracellular system was measured using the fluorescent dye DCFH-DA in Raw 264.7 cells. CoQ_{10} at concentrations of 1, 10 and 100 μM inhibited 7.4±4.6%, 15.5±3.5% and 23.4±3.5% of silica-induced and 10.5±6.1%, 20.5±4.8% and 31.3±6.7% of zymosan-induced ROS generation, respectively [Choi *et al.* 2009]. CoQ_{10} resulted in a significant increase in dihydrotestosterone yields by 84.4% and 128.2% in periosteal fibroblasts and osteoblasts respectively, which is normally decreased with oxidative stress [Figuero *et al.* 2006]. Studies have shown that low doses of CoQ_{10} reduce oxidation and DNA double-strand breaks [Quiles *et al.* 2004]. CoQ_{10} at doses of 100 mg three times daily, was found to improve endothelium bound extracellular SOD (ecSOD) in a double-blind, randomized controlled study of 35 coronary artery disease patients [Tiano *et al.* 2007].

Although CoQ_{10} has shown to possess many activities other than antioxidant activity, poor bioavailability poses a great limitation in its clinical use [Bank *et al.* 2011]. Poor bioavailability of CoQ_{10} can be attributed to its physicochemical properties like crystallinity, lipophilicity and high molecular weight (863 g/mol) [Bhagavan and Chopra 2007]. CoQ_{10} is absorbed slowly from the gastrointestinal tract, attributed to its physicochemical properties [Table 1.1]. At a daily dose of 90 mg/day in adults, about 3% of the administered dose is found in the blood. Being lipidic in nature, the absorption from the gastrointestinal tract follows the same process as that of other lipids and the uptake mechanism appears to be similar to that of vitamin E. It is better absorbed when taken with fatty food. After oral absorption or intravenous administration, CoQ_{10} is taken up by chylomicrons. Most of the exogenous dose is distributed to the liver and incorporated into very low density lipoprotein (VLDL). After incorporation

into VLDL in the liver, CoQ_{10} is subsequently concentrated in various tissues, including the adrenals, spleen, kidneys, lungs, and myocardium [Bhagavan and Chopra 2006]. The plasma peak can be observed 2-6 h after oral administration, mainly depending on the design of the study. In some studies, a second plasma peak was also observed at about 24 h after administration, probably due to both enterohepatic recycling and redistribution from the liver to circulation [Bhagavan and Chopra 2006]. Pharmacokinetic study with deuterium-labelled crystalline CoQ_{10} in humans determined an elimination half-time of 33 h [Tomono *et al.* 1986]. CoQ_{10} is metabolised in all the tissues and the metabolites are phosphorylated and transferred to kidneys. Urinary excretion of CoQ_{10} is very low and the major route for its elimination is biliary and faecal excretion [Ozawa *et al.* 1986]. The main metabolite of CoQ_{10} was a glucuronide of Q acid I [2,3 dimethoxy-5-methyl-6-(30-methyl-50-carboxy-2-pentenyl)-1,4-benzohydroquinone] formed in the liver [Kishi *et al.* 1964].

In order to improve the bioavailability of CoQ_{10} after oral administration, several new approaches have been considered and different new types of formulations have been developed and tested in animals or humans [Zmitek *et al.* 2008a]. The aqueous suspension of finely powdered CoQ_{10} in pure water, lecithin stabilised soybean oil microspheres containing CoQ_{10} [Ozawa *et al.* 1986], self emulsifying drug delivery system of CoQ_{10} [Kommuru *et al.* 2001] were developed in this way. A general rule is that the higher the dose that is orally administered, the lower is the percent of the dose absorbed for solubilised CoQ_{10} formulations [Bhagavan and Chopra 2007]. Increasing the solubility of the active agent in water using different pharmaceutical strategies facilitates increased absorption through GI tract. Various approaches have been developed to achieve this goal, with many of

them producing significantly better absorption [Zmitek *et al.* 2008b]. Some of the pharmaceutical approaches include the use of the aqueous dispersion of solid CoQ_{10} with tyloxapol polymer [Westesen and Siekmann 2001], formulations based on various solubilising agents [Ohashi *et al.* 1984] and microencapsulation into carbohydrate matrices [Bule *et al.* 2010]. Encapsulation of active moieties into polymeric matrices is an approach adapted these days to improve the bioavailability and many types of micro and nanoparticles of CoQ_{10} have been formulated for this purpose [Hsu *et al.* 2003, Ankola *et al.* 2007, Hu *et al.* 2011].

Previously, CoQ_{10} loaded poly(lactide-co-glycolic) acid (PLGA) nanoparticles were developed in our laboratory. These nanoparticles showed increased uptake from the rat intestine. At 60% reduced dose than the suspension formulation, nanoparticles showed increased efficacy in treating hypertension, suggesting improved bioavailability and efficacy [Ankola *et al.* 2007].

1.1.3. Curcumin

Curcumin is a natural compound found in the rhizome of *Curcuma* species. Curcumin exists in equilibrium with its enol tautomer. The bis-keto form predominates in acidic and neutral aqueous solutions. Above pH 8, the enolate form of the heptadienone chain predominates [Fig. 1.1A]. Curcumin acts mainly as an electron donor, a mechanism more typical for the scavenging activity of phenolic antioxidants [Shen and Ji 2007]. Presence of diketone moiety is essential in the radical scavenging activity of curcumin [Tcnnesen and Greenhill 1992].

Curcumin had an effective DPPH scavenging, ABTS scavenging, N,N-dimethyl-p-phenylenediamine dihydrochloride (DMPD) scavenging, superoxide anion radical scavenging, H_2O_2 scavenging, ferric ions (Fe^{3+})

reducing power and ferrous ion (Fe^{2+}) chelating activities. Curcumin inhibited 97.3% lipid peroxidation of linoleic acid emulsion at 20 mM concentration, where as to show the similar activity butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), vitamin E and trolox required 123, 102, 51 and 90 mM concentrations respectively [Ak and Gulcin 2008].

A.

B.

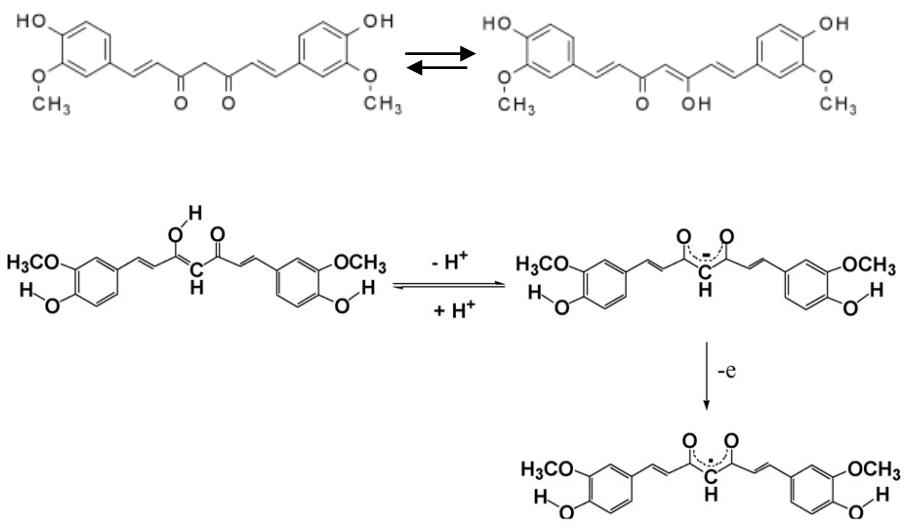


Fig 1.1. Curcumin tautomerism (A), Sequential proton loss and electron transfer radical scavenging activity of curcumin (B) [Shen and Ji 2007]

Phenolic antioxidants like curcumin, usually scavenge free radicals by an electron-transfer mechanism [Fig. 1.1B]. Antioxidant mechanisms of curcumin have been studied by laser flash photolysis and pulse radiolysis [Jovanovic *et al.* 2001]. Results indicate that one of the curcumin oxyl radicals generated by its antioxidant action undergoes molecular reorganization, i.e., the initially generated curcumin β-oxo alkyl is transformed into the phenoxyl radical. This phenoxyl radical can be repaired by water soluble antioxidants to regenerate curcumin. Pure curcumin is thought to position itself within the cell membrane, where it intercepts lipid (peroxyl) radicals and becomes a phenoxyl radical. Being more polar than curcumin, the phenoxyl radical may

move to the surface of the membrane, where it may be regenerated by any water-soluble antioxidant. Curcumin efficiently and rapidly reacts with the methyl propionamidine peroxyl radicals, which are the model for lipid peroxyl radicals. Such antioxidant action of curcumin may be expected from the favourable reduction potential of the phenol moiety [Jovanovic *et al.* 2001].

Curcumin, being an antioxidant, inhibits lipid peroxidation in rat liver microsomes, erythrocyte membrane and brain homogenates [Reddy and Lokesh 1994]. Curcumin can lower lipid peroxidation by maintaining the activation of antioxidant enzymes like SOD, catalase and GPx at higher levels. Curcumin is capable of scavenging oxygen free radicals, such as superoxide anions and hydroxyl radicals [Reddy and Lokesh 1992]. Addition of curcumin (5 µM) to a mixture of astrocytes and oligodendrocytes (C6 rat glioma cells) significantly modulates gene expression in four primary pathways: oxidative stress, cell cycle control, DNA transcription and metabolism. Several genes like aldo-keto reductase, glucose-6-phosphate dehydrogenase, and aldehyde oxidase that protect against oxidative stress were also identified to be up- or downregulated by curcumin [Panchal *et al.* 2008]. Seven days of oral curcumin supplementation (3.6 g/day) decreased the number of oxidative DNA adducts in malignant colorectal tissue in humans. Curcumin taken orally may be effective on the gastrointestinal walls to inhibit oxidative DNA damage [Sharma *et al.* 2004, Garcea *et al.* 2005]. In addition to direct antioxidant activity, curcumin may function indirectly as an antioxidant by inhibiting the activity of inflammatory enzymes or by enhancing the synthesis of GSH.

Being a photosensitive compound, about 5% decrease in absorbance of curcumin has been measured during the time for typical sample preparation

when clear rather than amber glassware is used. Due to the light sensitivity curcumin should be protected from light. Curcumin decomposes when exposed to sunlight, both in ethanolic and methanolic extracts and in neutral-basic pH conditions and as a solid. The stability of curcumin can be strongly improved by lowering the pH or by adding GSH, NAC or ascorbic acid. The increased stability of curcumin in acidic pH condition may be due to the prevalence of the conjugated diene structure. The point of failure for the clinical development of oral curcumin is due to its very low bioavailability which is attributed to its poor solubility, instability and first pass metabolism [Wang *et al.* 1997].

Due to its limited oral bioavailability in humans, plasma and tissue concentrations of curcumin are likely to be very low. Low serum levels of curcumin may be due in part to the extensive intestinal and hepatic metabolic biotransformation [Sharma *et al.* 2007]. When administered orally (250 mg/kg), curcumin reached the maximum concentration (90 ng/ml) at 0.5 h, and declined below the detection limit within 6 h in rats [Shaikh *et al.* 2007]. Curcumin undergoes extensive metabolism by conjugation and reduction [Sharma *et al.* 2007]. Curcumin is readily conjugated in the intestine and liver to form curcumin glucuronides and curcumin sulfates or reduced to hexahydrocurcumin. Curcumin metabolites may not have the same biological activity as the parent compound. Conjugated or reduced metabolites of curcumin were less effective inhibitors of inflammatory enzyme expression in cultured human colon cells than curcumin itself [Ireson *et al.* 2001].

In a clinical trial conducted in Taiwan, serum curcumin concentrations peaked 1-2 hours after an oral dose, and peak serum concentrations were 0.5, 0.6 and 1.8 µM/L at doses of 4, 6 and 8 g/day, respectively [Ireson *et al.* 2002].

Another clinical trial conducted in the UK found that plasma curcumin, curcumin sulfate and curcumin glucuronide concentrations were in the range of 10 nmol/L one hour after a 3.6 g of oral curcumin [Sharma *et al.* 2004]. There is some evidence that orally administered curcumin accumulates in gastrointestinal tissues. When colorectal cancer patients took 3.6 g/d of curcumin orally for 7 days prior to surgery, curcumin was detected in malignant and normal colorectal tissue [Garcea *et al.* 2005]. In contrast, curcumin was not detected in the liver tissue of patients with liver metastases of colorectal cancer after the same dose of oral curcumin. Oral administration of curcumin may not effectively deliver curcumin to tissues other than the gastrointestinal tract [Garcea *et al.* 2004]. The poor absorption from the intestine, coupled with the high degree of metabolism of curcumin in the liver and its rapid elimination through bile, result in short residence times in tissues [Pan *et al.* 1999].

Many drug delivery approaches have come up to overcome the bioavailability problem and to prove curcumin's activity in different diseases. Micronised suspension, nanosuspension, solid dispersion, inclusion complexes, self emulsifying drug delivery systems, phospholipid complexes and polymeric nanoparticles were explored for the delivery of curcumin [Bisht *et al.* 2010, Setthacheewakul *et al.* 2010, Munjal *et al.* 2011]. One such delivery system is the use of phytosomes, complex of curcumin with phosphatidylcholine (Meriva®). This formulation was superior in terms of hydrolytic stability, human pharmacokinetics and clinical study including 100 osteoarthritis patients [Belcaro *et al.* 2010]. Previously, we have developed curcumin encapsulated nanoparticles and found that the nanoparticles approach resulted in atleast 9 fold increase in the oral bioavailability in rats [Shaikh *et al.* 2009].

1.1.4. Our approach

Mixed results with antioxidants in oxidative stress related diseases led to critical analysis of the antioxidant trails in establishing the reasons behind the failure. The overwhelming failure of antioxidant therapy to in many diseases can be explained by inadequacy of the doses of antioxidants used, short duration of therapy, or poor timing in the initiation of the supplementation. Many researchers indicated the poor bioavailability as the reason [Belcaro *et al.* 2010], some pointed out wrong selection of cohort in the antioxidant trials [Halliwel, 2000].

The three antioxidants EA, CoQ_{10} and curcumin have a strong antioxidant potential and have some problems associated with stability and bioavailability as discussed. The involvement of free radicals in disease conditions like hyperlipidaemia, diabetes and pulmonary hypertension prompted us to explore the advantages of the above said antioxidants in these conditions, employing nanoparticle delivery approach to overcome biopharmaceutical hurdles.

1.2. Hyperlipidaemia

Hyperlipidaemia or dyslipidaemia is the presence of raised or abnormal levels of lipids and/or lipoproteins in the blood. Elevated cholesterol levels in the blood are linked with increased risk of atherogenic diseases. LDL particles play an important role in the atherogenic diseases as these particles contain more than 50% of the total cholesterol [Jain *et al.* 2007].

The risk factors of atherosclerosis include cigarette smoking, hypertension, low levels of high density lipoprotein cholesterol (HDL-C), high levels of LDL-C, high levels of very low density lipoprotein cholesterol (VLDL-C), family history of premature coronary heart disease (CHD) and

age [Cooper *et al.* 2008]. Research suggests that LDL-C lowering reduces the incidence of CHD [Steinberg 2006a]. Thus, cholesterol lowering therapy is the primary target in patients who are at risk of developing cardiovascular diseases. The basic principle of prevention is to adjust the intensity of risk reduction therapy with respect to the absolute risk. Depending upon the number of risks associated with the patient the goal of LDL-cholesterol lowering is set and the same factors also influence the choice of drug therapy with therapeutic lifestyle changes.

1.2.1. Oxidative stress and hyperlipidaemia

Hyperlipidaemia and oxidative stress often co-exist and their collective effect increases the risk of atherosclerosis [Kostapanos *et al.* 2011]. Oxidative stress, mostly by giving rise to endothelial dysfunction and pro-inflammatory processes, plays a major role in the atherogenesis [Madamanchi *et al.* 2005]. One of the initial events in the development of atherosclerosis is the accumulation of cells containing excess lipids within the arterial wall. In addition, increased intracellular generation of ROS plays an important role in the chronic inflammatory response to atherosclerosis [Chisolm and Steinberg 2001].

Some of the oxidative stress related parameters were found to be high including TBARS and carbonyl protein levels in patients with high cholesterol levels. It was demonstrated that SOD and GSH activities were decreased in hypercholesterolaemic subjects. Significant correlation between total cholesterol, high sensitivity C- reactive protein (hs-CRP), and oxidative stress biomarkers is observed in hypercholesterolaemic subjects. Hypercholesterolemia elicits a cascade of pro-inflammatory reactions that are known to lead to the production of ROS [Duarte *et al.* 2010]. Lipid and water

soluble antioxidants are lower and lipid peroxide content and LDL oxidation susceptibility are higher in hypercholesterolaemic patients [Moriel *et al.* 2000]. An early stage of hyperlipidaemia represents increase in lipid peroxidation and decreased antioxidant enzyme activity. Advanced stage of the hyperlipidaemia represents excess production of lipid peroxidation products, oxidative modification of LDL, protein glycation, glucose-auto-oxidation [Yang *et al.* 2008].

1.2.2. Cholesterol transport

Cholesterol is hydrophobic and the transport of soluble cholesterol in blood is extremely low. Cholesterol and triglycerides are transported in lipoprotein particles. The external surfaces of the lipoprotein particles are hydrophilic and the internal surface is lipophilic where the cholesterol and triglycerides can reside and transported across the blood stream. Lipoproteins also contain signalling moieties on their surfaces which help in delivering the cholesterol and triglycerides to specific cell types. Lipoprotein particles are categorized based on their densities based on the amounts of cholesterol, triglycerides and apolipoproteins. The densest and smallest are called HDL, the least dense, and the largest of the lipoproteins, are the chylomicrons. After chylomicrons, VLDL, intermediate density lipoproteins (IDL), LDL and HDL can be arranged in the descending order of size and ascending order of density. LDL particles transport the majority of cholesterol and are responsible for the transport of cholesterol to the peripheral tissues. HDL plays a crucial role in reverse transport of cholesterol from peripheral tissues to the liver [Fig. 1.2].

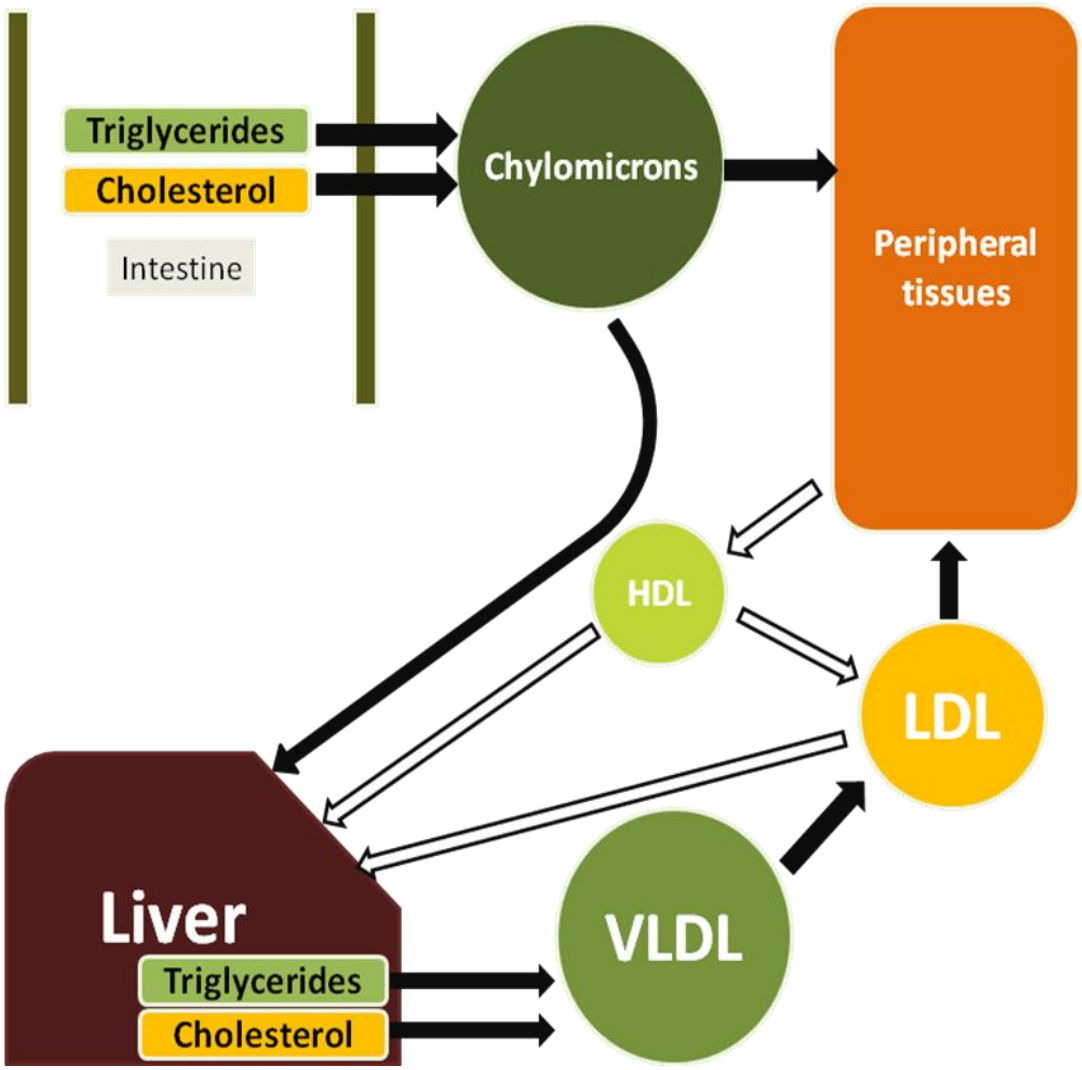


Fig 1.2: Cholesterol transport in humans. Cholesterol and triglycerides are absorbed from the intestine and transported through chylomicrons to the peripheral tissues and to liver as chylomicron remnants. During blood circulation chylomicrons exchange lipids and proteins with HDL particles to form chylomicron remnants which are broken down in liver. VLDL particles carry the cholesterol synthesised in liver and triglycerides from liver and during transport they convert to LDL particles which are rich in cholesterol and transport cholesterol to the peripheral tissues. Empty arrows show the reverse transport of cholesterol from peripheral tissues to the liver.

Chylomicrons are generated in the intestinal walls and transport fats from the intestine to muscle and other tissues. These are the exogenous cholesterol and triglyceride transporters. After transporting the cholesterol and triglycerides to tissues chylomicrons circulate back to liver, which are rich in cholesterol and are called chylomicron remnants. The endogenous transporters of cholesterol to peripheral tissues are VLDL particles. These are produced by the liver and contain triacylglycerol and cholesterol. During the transport in the bloodstream VLDL particles lose triacylglycerol to leave IDL particles which contain higher percentage of cholesterol. The IDL molecules are either taken up by the liver for metabolism into other biomolecules or continue to lose triacylglycerols in the bloodstream until they form LDL particles.

1.2.3. Classification of hyperlipidaemia and treatment

Hyperlipidaemia is generally associated with lifestyle and genetic causes. Classifying the hyperlipidaemia based on any specific aetiology is cumbersome as there is an overlap of genetic and lifestyle changes in many of the conditions. Fredrickson's classification is the most acceptable classification of hyperlipidaemia. Fredrickson classification is based on the pattern of lipoproteins on electrophoresis or ultracentrifugation [Table 1.2]. This system is adopted by the world health organisation (WHO) and it does not distinguish among the types of genes responsible for the aetiology. Reduction in blood lipid levels is achieved often by combining dietary modifications and cholesterol lowering drugs.

1.2.3.1. Dietary modifications

Therapeutic lifestyle changes are recommended for LDL-C lowering therapy at the initial stages of hyperlipidaemia. It mainly includes reduction in the intake of food rich in fat content and increased energy expenditure by

increasing physical activity. Some specific guidelines for regular food habits were provided by adult treatment panel III of national cholesterol education program (NCEP) in US [Table 1.3]. In the UK, National Institute for Health and Clinical Excellence (NICE) has made similar recommendations for the management of lipid levels [Cooper *et al.* 2008]. The following foods or nutraceuticals, such as dietary fiber, phytosterols, soybean, hawthorn fruit, tea, fermented dairy products, policosanols, buckwheat, oats, rice bran oil, onion, garlic, red yeast rice, almond, fish oil, flaxseed, black rice, licorice and ginseng oil, have been claimed to possess cholesterol-lowering activity [Chen *et al.* 2011].

Table 1.2: Fredrickson classification of hyperlipidaemia and its regulation

Type	Synonyms	Treatment
Type I	Buerger-Gruetz syndrome, Primary hyperlipoproteinaemia, or Familial hyperchylomicronaemia	Diet control
Type IIa	Polygenic hypercholesterolaemia or Familial hypercholesterolaemia	Bile acid sequestrants, niacin, statins
Type IIb	Combined hyperlipidaemia	Statins, niacin, fibrates
Type III	Familial dysbetalipoproteinaemia	Fibrates, statins
Type IV	Familial hyperlipaemia	Fibrates, niacin, statins
Type V	Endogenous hypertriglyceridaemia	Niacin, fibrates

Table 1.3. Nutrient composition of the therapeutic life style changes diet

Nutrient	Recommended Intake
Saturated fat	Less than 7% of total calories
Polyunsaturated fat	Up to 10% of total calories
Monounsaturated fat	Up to 20% of total calories
Total fat	25-35% of total calories
Carbohydrate	50-60% of total calories
Fibre	20-30 g/day
Protein	Approximately 15% of total calories
Cholesterol	Less than 200 mg/day
Total calories (energy)	Balance energy intake and expenditure to maintain desirable body weight/prevent weight gain

Taken from adult treatment panel III, NCEP

1.2.3.2. Pharmacotherapy of hyperlipidaemia

The most effective drugs in reducing the risk of CHD are the statins [Lardizabal and Deedwania 2011]. Other classes of drugs include nicotinic acid (niacin), bile sequesterants and fibric acid derivatives [Table 1.4]. Statins are the most effective means of lowering LDL-C; nicotinic acid is the most effective in raising HDL-C, and fibrates in lowering triglycerides. Ezetimibe is relatively ineffective in lowering LDL when given as monotherapy but is of great therapeutic value as an adjunct to statins in the treatment of severe hypercholesterolaemia, with fewer side effects. If monotherapy with one of the classes of drugs does not lower LDL-C to the required extent to reduce the CHD risk, combination therapy is applied. In case of management of dyslipidaemias combination of drugs is always preferred. Addition of ezetemibe to the therapy reduces the levels of LDL-C very effectively. In severe familial hyperlipidaemia even maximal doses of statins do not always lower LDL-C sufficiently and an anion exchange resin is often added. In

mixed hyperlipidaemia, statin monotherapy may fail to reduce triglycerides and raise HDL-C to the desired levels and it may be necessary to add either nicotinic acid or a fibrate to achieve these objectives [Thompson 2004].

Table 1.4: Drugs used in the treatment of hyperlipidaemia

Drug	Specific use	Adverse effects
Statins	Hypercholesterolaemia	Myopathy, Increased liver enzymes
Niacin	Hypercholesterolaemia & hypertriglyceridaemia	Flushing, Hyperglycaemia, Hyperuricaemia, Upper GI Distress, Hepatotoxicity
Fibrates	Hypercholesterolaemia & hypertriglyceridaemia	Dyspepsia, Gallstones, Myopathy, Unexplained non-CHD deaths
Bile acid sequestrants	Hypercholesterolaemia & hypertriglyceridaemia	GI distress, Constipation, Decreased absorption of other drugs
Ezetemibe	Hypercholesterolaemia	No known severe adverse effects

Cholesterol lowering therapy is a preventive measure to reduce the risk of CHD and it requires a long term usage of LDL-C lowering drugs. Aggressive control of LDL-C with high doses or long term clinical usage is associated with serious adverse effects in some cases. The treatment for hyperlipidaemia in the initial stages starts with life style changes, functional foods and nutraceuticals. As hyperlipidaemia advances physicians start to introduce pharmacotherapy and give less importance to nutraceuticals. Although nutraceuticals show benefits alone or in combination with pharmaceuticals they are given less importance at the later stages of hyperlipidaemia. Nutraceuticals delivered in efficacious delivery systems

must be given equal preference with the pharmaceuticals in treating hyperlipidaemia [Marinangeli and Jones, 2010].

1.2.4. Antioxidants in hyperlipidaemia

Antioxidants in hyperlipidaemia not only attenuate the oxidative stress but they also alleviate the increased levels of lipids. Although the understanding of the role of antioxidants on the levels of cholesterol and triglycerides is poor, some researchers suggest inhibition of cholesterol absorption as the mechanism for antihyperlipidaemic activities of antioxidants [Lei *et al.* 2007]. There is an increasing amount of evidence on the secondary messenger activities of antioxidants in gene expression. Based on this some suggest that the antioxidants may affect the activity of lipoprotein lipase directly and/or indirectly by scavenging ROS, which in turn influences lipid metabolism [Yang *et al.* 2006].

Thymoquinone, an herbal antioxidant, attenuated the aortic MDA levels and atherosclerosis in ciclosporine A and high cholesterol diet induced hyperlipidaemia [Ragheb *et al.* 2011]. Administration of lipoic acid and NAC to high fat diet (HFD)-fed rats prevented the rise in oxidative stress by restoring SOD and GSH in the serum and increased the LPL activity [Yang *et al.* 2006].

Hyperlipidaemia is associated with endothelial dysfunction, an early event in atherosclerosis and predictor of risk for future coronary artery disease. Early detection and treatment of endothelial dysfunction in high-risk children may retard the progression of atherosclerosis. Antioxidant therapy with vitamins C and E restores endothelial function in hyperlipidaemic children [Engler *et al.* 2003]. Antioxidants decrease activation of redox-transcription factors and increase endothelial nitric oxide synthase (eNOS)

expression in human endothelial cells and atherosclerotic prone areas in hypercholesterolaemic LDL receptor knockout mice [Nigris *et al.* 2005].

1.2.5. Ellagic acid and CoQ_{10} in hyperlipidaemia

EA has been shown to possess antihyperlipidaemic actions in rats [Lei *et al.* 2007] Supplementation with EA (1% w/w of diet) reduced serum triglycerides (TG) and total cholesterol (TC) in rabbits receiving atherogenic diet. Apart from this EA also shown reduction in lipid peroxidation, oxidative DNA damage and lipid lesions in the histological examination [Yu *et al.* 2005].

CoQ_{10} reduced the elevated levels of TBARS, MDA and increased the levels of vitamin E in transfatty acid rich diet fed rats [Singh *et al.* 2000]. . Addition of CoQ_{10} to the fenofibrate has shown to be efficacious in reducing the massive hypertriglyceridaemia (MHTG) in patients who were previously non-responsive to fenofibrate. However, CoQ_{10} supplementation alone has shown no improvements in the MHTG. Even though the mechanism is unknown these results showed that CoQ_{10} is beneficial in lipid lowering [Cicero *et al.* 2005]. Physiological levels of CoQ_{10} are reduced to below normal in statin induced myopathy and supplementation of CoQ_{10} in such conditions is thought to be beneficial [Rundek *et al.* 2004, Caso *et al.* 2007].

1.3. Diabetes and its complications

Type 1 and type 2 diabetes mellitus (T1D, T2D) have high blood glucose levels (hyperglycaemia) in common which can cause serious complications of the vasculature in the advanced stages of the disease [Haan *et al.* 2011]. Clinical signs such as excessive thirst, urination, and hunger are the primary symptoms to diagnose diabetes mellitus [Belle *et al.* 2011]. T1D is the extensive loss of pancreatic β-cells and in T2D there is almost 50%

reduction in the β-cell volume. Autoimmunity is the predominant effector mechanism of T1D, but may not be its primary cause. T1D precipitates in genetically susceptible individuals, very likely as a result of an environmental trigger [Atkinson & Eisenbarth 2001]. T1D usually starts in people younger than 30 and is therefore also termed juvenile-onset diabetes, even though it can occur at any age. T2D is mostly the result of insulin resistance which is usually associated with obesity or older age. In the case of insulin resistance the muscle or adipose cells do not respond adequately to normal levels of insulin produced by intact β-cells. Overall life expectancy for patients with diabetes mellitus is ~7–10 yrs shorter than people without diabetes mellitus [Belle *et al.* 2011]. The burden of diabetes is mostly due to its severe complications. Long-term complications of diabetes mellitus can be classified into 3 major types: macrovascular disease, microvascular disease, and endocrine complications [Kassab *et al.* 2001]**.**

1.3.1. Oxidative stress and diabetes

Oxidative stress has been implicated in both the onset and the progression of diabetes and its associated complications [Haan *et al.* 2011]. Some of the consequences of oxidative stress are the development of insulin resistance, β-cell dysfunction, impaired glucose tolerance, and mitochondrial dysfunction, which can lead to diabetes. Experimental and clinical data suggest an inverse association between insulin sensitivity and ROS levels. Hyperglycaemia can cause an increase in oxidative stress and membrane lipid peroxidation [Rains and Jain 2011]. Increased mitochondrial ROS generation is known to be implicated in the development of diabetic complications [Nishikawa *et al.* 2000]. Other potential sources of ROS production in diabetes include NADPH oxidases (NOX), Xanthine oxidase

(XO), nitric oxide synthase (NOS), lipoxygenase and cytochrome P450 mono-oxygenases [Guzik *et al.* 2002, Sorescu and Griendling 2002].

The polyol pathway, increases in advanced glycation end products (AGEs), activation of protein kinase C and increases in hexosamine flux produce excess amounts of free radicals under hyperglycaemia [Haan *et al.* 2011]. Antioxidant defense mechanisms are inherent in cells and tissues, which aid in preventing the accumulation of ROS. However, in diabetes the antioxidant defense system weakens [Sekhar *et al.* 2011]. Glycation of antioxidative enzymes due to hyperglycaemia impairs cellular defense mechanisms, leading to the development of diabetic complications [Kang 2003]. F2-isoprostane levels have been reported to be increased in the plasma of T2D and in the urine of T2D and T1D subjects [Gopaul *et al.* 1995]. Moreover, correlation between impaired glycaemic control and enhanced lipid peroxidation has been reported in diabetes [Davy *et al.* 1999].

1.3.2. Antioxidants in diabetes

Antioxidant therapy has been of great interest as a way to combat oxidative stress in diabetic patients over the past decade. Although a very logical approach, it requires more than simple dosing with an antioxidant. Antioxidants that target mitochondrial ROS may offer an alternative approach to reduce diabetes-driven oxidative stress since it is becoming increasingly clear that mitochondria play a vital role in diabetes-mediated ROS production [Nishikawa *et al.* 2000]. Furthermore, targeting other sources of ROS, for example, NOX inhibitors, is yet another approach being explored by several laboratories [Haan *et al.* 2011].

Vitamin C (ascorbic acid) prevented the protein glycation to protect the endothelium in diabetic rats [Riccioni *et al.* 2007]. However, in humans, vitamin C has not been shown to improve endothelial dysfunction or insulin

resistance [Chen *et al.* 2006]. Vitamin E showed improvement in insulin sensitivity and secretion in diabetic patients [Costacou *et al.* 2008]. α-lipoic acid, anotherantioxidant has been shown to normalise the fructose induced diabetes in rats by preserving the β-cell function [Cummings *et al.* 2010]. α-lipoic acid also shown beneficial effects in diabetic apo E knockout mice by protecting the β-cell function, reducing cholesterol levels and preventing diabetic nephropathy [Yi *et al.* 2011]. 900 mg of NAC twice daily for 4 weeks resulted in decrease in CRP levels in diabetic patients. However it does not had any effect on the endothelial function in diabetics [Jeremias *et al.* 2009].

1.3.3. CoQ_{10} and curcumin in diabetes and its complications

Human umbilical vein endothelial cells (HUVEC) exposed to high concentrations (30 mM) of glucose exhibited abnormal properties like morphological and biochemical features of apoptosis, excessive production of ROS and increased expression of eNOS. Treatment with CoQ_{10} in HUVEC prevented all these changes under high glucose condition [Tsuneki *et al.* 2007]. Treatment with CoQ_{10} lowered glycated haemoglobin (HbA1c) and lipid peroxidation in Goto-Kakizaki (GK) rats. The spontaneously diabetic GK rat is a genetic non-obese type 2 diabetes model that is characterized by a deficient insulin response to glucose *in vivo* and *in vitro*. GK rats had diabetes at 4 weeks of age, characterized by higher basal plasma glucose and insulin levels compared to Wistar rats. When CoQ_{10} was administered intraperitonially 3 days a week (for 8 weeks) at a dose of 20 mg/kg body weight resulted in decreased HbA1c levels and increased antioxidant levels [Sena *et al.* 2008].

Generation of oxidative stress is implicated in STZ induced pancreatic islet cell toxicity. In an *in vitro* study, isolated islets from C57/BL6J mice were incubated with curcumin for 24 h and later exposed to STZ for 8 h. Islet

viability and secreted insulin in curcumin pre-treated islets were significantly higher than islets exposed to STZ alone. Curcumin retarded generation of islet ROS, and it prevented reduction in levels of cellular free radical scavenging enzymes, although it did not cause overexpression of Cu/Zn SOD [Meghana *et al.* 2007].

In STZ induced diabetic rats treatment with oral curcumin (1g/kg body weight) showed significant hypoglycaemic activity compared with the diabetic group. In STZ treated rats retinal GSH levels were decreased by 1.5-fold, and antioxidant enzymes, SOD and catalase, showed decrease in activity in the diabetic group. Curcumin positively modulated the antioxidant defense system. Proinflammatory cytokines, TNF-α and vascular endothelial growth factor (VEGF) were elevated in the diabetic retinae, but were normalised by curcumin [Gupta *et al.* 2011]. Curcumin was also able to inhibit the aldose reductase and reduce the accumulation of sorbitol in human erythrocytes under high glucose conditions [Muthenna *et al.* 2009].

1.4. Pulmonary hypertension

Pulmonary hypertension (PH) is an increase in blood pressure in the lung vasculature (i.e. pulmonary artery, pulmonary vein, or pulmonary capillaries). PH is characterised by shortness of breath, dizziness and fainting. PH is a severe disease with markedly decreased exercise tolerance and right- or left-sided heart failure. It can be one of the five different types based on the cause and therapeutic strategy: arterial, venous, hypoxic, thromboembolic, or miscellaneous. PH is a complex condition associated with vascular remodelling in the pulmonary circulation, either as a primary intrinsic defect or as a secondary response to hemodynamic changes associated with cardiac defects, pulmonary embolus, hypoxic conditions or co-morbid conditions.

1.4.1. Epidemiology, aetiology & pathophysiology

Epidemiological data is obtained from prospective registries in the United States and European countries. In the French registry, around half of 674 pulmonary arterial hypertension (PAH) cases were reported in patients with co-morbid conditions. The other half had no identifiable risk factor, corresponding to idiopathic (sporadic) or familial PAH. Swiss registry for PH reported that, the incidence was 15 cases per million [Tueller *et al.* 2008]. In Scotland, the national hospitalization records show a prevalence of 52 cases per million [Humbert 2008]. Women are affected more commonly than men, and patients of all ages may develop PH. The mean age at diagnosis has been reported at 36 to 50 yrs.

An imbalance may exist between vasodilators and vasoconstrictors as well as between growth inhibitors and mitogenic factors in PH [Vincent *et al.* 1993, Tuder *et al.* 1999, Black and Fineman 2006, Chan and Loscalzo 2008]. Other than these mediators, oxidative stress has also been implicated in the pathogenesis of PH [Sanders and Hoidal 2007].

Prostacyclin and thromboxane A_2 are major arachidonic acid metabolites of endothelial cells and to some extent smooth muscle cells. Prostacyclin is a potent vasodilator, inhibits platelet aggregation, and has antiproliferative properties; in contrast, thromboxane A2 is a potent vasoconstrictor. In PH, the balance between these two molecules is shifted towards thromboxane A_2. Furthermore, the expression of prostacyclin synthase, the enzyme responsible for synthesis of prostacyclin (PGI_2), is decreased in the small and medium-sized pulmonary arteries of idiopathic PAH patients [Tuder *et al.* 1999]. Transgenic mice that express rat prostacyclin synthase in the distal lung epithelium developed no PH after exposure to hypobaric hypoxia. From this it can be understood that PGI_2 can

play a major role in regulating pulmonary vascular tone in response to hypoxia and can modify vascular remodelling [Geraci *et al.* 1999].

Endothelin-1 (ET-1) is a potent vasoconstrictor and is mitogenic in pulmonary arterial smooth muscle cells (PASMC) [Hassoun *et al.* 1992]. Increased levels of ET-1 have been found in rat models of PH as well as in the plasma and the pulmonary vascular wall of patients with idiopathic PAH and other forms of PH [Giaid *et al.* 1993]. This increase in ET-1 levels is proportional to the magnitude of pulmonary blood flow and cardiac output [Vincent *et al.* 1993]. Although its production and actions are predominantly local, circulating levels of ET-1 are increased in PAH and correlate with disease severity [Giaid *et al.* 1993].

Nitric oxide (NO) is a potent vasodilator, an inhibitor of platelet activation and vascular smooth muscle cell proliferation. Upregulation of endothelial nitric oxide synthase (eNOS) was observed in infants with persistent pulmonary hypertension (PPHN) [Sood *et al.* 2011]. eNOS is increased in the plexiform lesions of idiopathic PAH, where it could promote pulmonary endothelial cell proliferation [Mason *et al.* 1998]. In resistance pulmonary arteries, there was a progressive increase in nitrotyrosine (a measure of nitrative stress), which matched the increase in eNOS. Nitrotyrosine is also increased in conduit pulmonary arteries, but only after 14 days of hypoxia in rats [Demiryurek *et al.* 2000].

The serotonin (5-hydroxytryptamine or 5-HT) signalling pathway has also been implicated as a potential causative factor in PAH [Eddahibi and Adnot 2006]. Serotonin is both a vasoconstrictor and mitogen that promotes smooth muscle hyperplasia and hypertrophy. Primarily stored in platelet granules, secreted serotonin binds to its receptors present on PASMCs. Activation of these receptors leads to a decrease in adenylyl cyclase and

cyclic AMP, resulting in an increase in contraction. Furthermore, the cell-surface serotonin transporter (5-HTT) allows for transport of extracellular serotonin into the cytoplasm of smooth muscle cells, thereby activating cellular proliferation directly through the action of serotonin or indirectly via potential pleiotropic mechanisms [Chan and Loscalzo 2008].

Adrenomedullin dilates pulmonary vessels, increases pulmonary blood flow [Nicholls *et al.* 2001]. Plasma levels of adrenomedullin increase in proportion as a compensatory mechanism to PH [Nishikimi *et al.* 1997]. In addition, chronic infusion of adrenomedullin attenuates PH and medial thickening of pulmonary arterioles in rats treated with monocrotaline (MCT) [Yoshihara *et al.* 1998]. Vasoactive intestinal peptide, a potent systemic vasodilator decreases pulmonary artery pressure and pulmonary vascular resistance in rabbits with MCT-induced PH and in healthy human subjects [Soderman *et al.* 1993]; it also inhibits the vascular smooth muscle cell proliferation [Maruno *et al.* 1995].

Vascular changes that have been observed in PH include vasoconstriction, cellular proliferation, thrombosis and vascular remodelling. Pulmonary vascular remodelling appears because of the thickening of the arterial wall and increased resistance causes the vessel walls to intrude into the lumen and reduce its diameter. These structural changes include muscularisation of previously non-muscular arterioles, increased medial thickness of previously partially and completely muscular arterioles, adventitial hypertrophy and deposition of additional matrix components in the vascular walls. Due to the increased resistance from the pulmonary vasculature the pulmonary arterial pressure increases and with time leads to cardiac hypertrophy and death.

1.4.2. Oxidative stress in pulmonary hypertension

Superoxide generation was enhanced when pulmonary arterial pressure was elevated. Development of PH in lambs, with surgically created heart defect and increased pulmonary blood flow (Shunt), is associated with increases in oxidative stress. Increased expression and activity of NADPH oxidase (NOX) complex is responsible for the oxidative stress in lambs with PH [Grobe *et al.* 2006]. ROS generating systems stimulate PASMC proliferation [Sanders and Hoidal 2007]. In fetal lambs ROS generated through NOX is demonstrated to participate in the pathogenesis of the PH [Brennan *et al.* 2003]. Chronic hypoxia increases ROS levels and impairs endothelial NO-dependent relaxation in mice pulmonary arteries [Fresquet *et al.* 2006]. Reduced antioxidant status due to decreased SOD and GPx activities in the endothelial cells is also believed to be involved in the pathogenesis of PH [Masri *et al.* 2008].

Nitrotyrosine, a footprint of peroxynitrite is up-regulated in the lungs of infants with PH [Wadsworth *et al.* 2004], and in patients with advanced stage of PH [Bowers *et al.* 2004]. Peroxynitrite causes mitochondrial dysfunction leading to excessive ROS production [Crosswhite and Sun 2010], and pulmonary arterial smooth muscle and endothelial cell proliferation [Agbani *et al.* 2011]. Nitrotyrosine is also produced in pulmonary arteries of rats correlating with the development of PH in hypoxic environment [Demiryürek *et al.* 2000].

ROS generated by xanthine plus xanthine oxidase (X+XO) caused significant vasoconstriction in cannulated, pressurised resistance pulmonary arteries (PRA) of hypoxic piglets. Thromboxane synthase expression was increased in PRA from hypoxic piglets while PGI_2 synthase expression was similar in PRA from hypoxic and control piglets. Under conditions of chronic

hypoxia, altered vascular responses to ROS may contribute to PH by a mechanism that involves the prostanoid vasoconstrictor, thromboxane [Fike *et al.* 2011].

1.4.3. Treatment

Pulmonary hypertension other than pulmonary venous hypertension is treated based on the functional assessment classification of WHO (Table 1.5). The current treatment is limited and only provides symptomatic relief due to poor understanding of the aetiology and pathogenesis of the disease. The goals of current treatment regimens are to provide relief from symptoms, improve quality of life and to prevent death [Ruan *et al.* 2010].

Table 1.5: World Health Organization functional assessment classification

Class I	Patients with PH but without resulting limitation of physical activity. Ordinary physical activity does not cause undue dyspnoea or fatigue, chest pain, or near syncope.
Class II	Patients with PH resulting in slight limitation of physical activity. They are comfortable at rest. Ordinary physical activity causes undue dyspnoea or fatigue, chest pain, or near syncope.
Class III	Patients with PH resulting in marked limitation of physical activity. They are comfortable at rest. Less than ordinary activity causes undue dyspnoea or fatigue, chest pain, or near syncope.
Class IV	Patients with PH with inability to carry out any physical activity without symptoms. These patients manifest signs of right heart failure. Dyspnoea and/or fatigue may even be present at rest. Discomfort is increased by any physical activity.

Pharmacologic agents used in the treatment of PH include calcium-channel blockers, prostanoids, endothelin antagonists, and phosphodiesterase type 5 inhibitors (Table 1.6). These agents all have pulmonary vasodilatory effects and all except calcium-channel blockers also

have antiproliferative properties [Badesch *et al.* 2007, Ruan *et al.* 2010]. Epoprostenol was the first Food and Drug Administration (FDA) approved therapy for PH among prostaglandins and it remains the treatment of choice for most functional class IV patients. Other prostacyclin analogues in clinical use are treprostinil, iloprost and beraprost. Bosentan, a dual (ET_A and ET_B) endothelin receptor antagonist, was strongly recommended for functional class III PH. Sitaxsentan and ambrisentan are selective ET_A receptor antagonists which are also used in PH. Sildenafil, a selective inhibitor of cyclic guanosine monophosphate (cGMP) specific phosphodiesterase type 5 (PDE5), is strongly recommended for functional class II and III patients. Sildenafil increases the effects of locally produced NO by inhibiting the breakdown of NO's second messenger, cGMP. This results in pulmonary vasodilatation and inhibition of smooth muscle cell growth [Badesch *et al.* 2007].

Combination therapy has been suggested for patients with signs of right heart failure, 6-min walk distance, and persistent functional class III or IV symptoms despite treatment with a single medication [Chin and Rubin 2008]. Anticoagulation with Warfarin has been shown to improve survival, and is recommended for patients with IPAH [Johnson *et al.* 2006] and patients with an indwelling catheter for the administration of an intravenous prostanoid, in the absence of contraindications [Badesch *et al.* 2007].

PAH can also be cured by atrial septostomy which relieves the pressure on the right side of the heart, but at the cost of lower oxygen levels in blood (hypoxia). Lung transplantation can also be done to cure PAH, but it leaves the patient with the complications of transplantation, and a post-surgical median survival of just over five years [Badesch *et al.* 2007].

Table 1.6: Current therapy in the management of pulmonary hypertension

Therapy	Major drugs	Specific use	Drawbacks
Calcium channel blockers	Nifedipine, Amlodipine	IPAH patients who are vasoreactive by Swan-Ganz catheter	Only 50% of patients maintain long-term response
Prostaglandins	Epoprostenol Iloprost Treprostinil Beraprost	Treatment of choice for most functional class IV patients, used as initial therapy for class II and III with other drugs	Continuous infusion and use of an indwelling central catheter
Endothelin receptor antagonists	Bosentan, Ambrisentan Sitaxsentan	Treatment of choice for functional class III patients	Increase in liver function tests
Phosphodiesterase 5 inhibitors	Sildenafil	Functional class II and III	-

1.4.4. Need for new treatments

Recent advances in the treatment of PH resulted in the identification of therapeutic targets and the development of medications for its management. However, PH remains a debilitating and progressive condition. Currently available therapies improve functional capacity and quality of life and may improve survival. However, the vast majority of patients are left with severe functional limitations in their daily activities [Preston 2008].

In preclinical research, scientists are trying to demonstrate the pathophysiology and efficacy of new drugs and delivery systems in PH. Apart from severe functional limitations, the current treatments are also associated with serious delivery related drawbacks in PH. Epoprostenol requires constant intravenous infusion which makes it a complicated therapy. The complications with epoprostenol therapy resulted in the development of related drugs which can be delivered through other routes of administration. Subcutaneous treprostinil is developed in this manner; however, the subcutaneous injection causes intolerable pain, requiring analgesics which are effective sometimes but not always. Iloprost, another analogue, is administered through inhalation. Its short duration of action limits the long term use. Beraprost sodium is the first chemically stable and orally active prostacyclin analogue. The serious drawback of beraprost is the possibility that its beneficial effects may be attenuated with time. Endothelin antagonists like bosentan and sitaxsentan are associated with serious hepatotoxicities and other toxicities which raise questions on their use. All the drugs developed for the treatment of PH have one or the other problems associated with drug delivery, dosing regimens, adverse effects, and/or complications apart from poor efficacy. However, they remain the treatment

options because they are the only means of alleviating life threatening conditions in PH [Badesch *et al.* 2007]. The major concern with current therapy of PH viewed as a whole is its limited efficacy unlike the therapy of hyperlipidaemia or diabetes. There is a great need of efficacious therapeutic strategies to be developed for the treatment of PH.

1.4.5. Antioxidants in pulmonary hypertension

Antioxidant treatments are gaining importance in the research as the free radicals are shown to be implicated in the pathophysiology of PH. There are conflicting results with the treatment of antioxidants in PH. Concomitant β-carotene treatment protected the lung parenchyma from the inflammatory reaction and the septal fibrosis in rats developing PH with monocrotaline (MCT). β-carotene treatment slightly reduced the thickening of the wall of small arteries and arterioles and did not prevent right ventricular hypertrophy [Baybutt and Molteni 1999]. Vitamin E has a protective role on the development of MCT induced PH in rats. A modest reduction of the right ventricular hypertrophy in these rats was observed with high-dose vitamin E (3.3g vitamin E/kg diet) treatment. However, this treatment could not prevent the vascular hypertrophy caused by MCT [Molteni *et al.* 2004].

Treatment with 20 g/l NAC in drinking water 7 days before and 7 days after the initiation of hypoxia (4 weeks) in rats showed significant reduction of the right ventricular hypertrophy from 0.510 to 0.430 [Lachmanova *et al.* 2005]. In another study, NAC treatment (1% solution in drinking water) from one day before hypoxic exposure reduced mean pulmonary arterial pressure (mPAP), right ventricular hypertrophy, and pulmonary vascular media thickening caused by 3 wk of normobaric hypoxic exposure. It also reduced the lung tissue levels of phosphatidylcholine hydroperoxide (PCOOH), a primary peroxidation product of

phosphatidylcholine. Similar treatment with a XO inhibitor, allopurinol, reduced the increase in PCOOH levels in lungs and attenuated mPAP, right ventricular hypertrophy, and pulmonary vascular media thickening in rats exposed to hypoxia for 3 weeks [Hoshikawa *et al.* 2001]. Other antioxidants, 21 aminosteroid (U-83836E) and dimethylthiourea were also shown to be beneficial in PH [Lai *et al.* 1998].

Tempol (membrane-permeable SOD mimetic), has been shown to prevent the development of PH in chronic hypoxic rats. Treatment with tempol (86 mg/kg/day in drinking water) normalized right ventricular systolic pressure and reduced right ventricular hypertrophy while the pulmonary vascular media thickening unchanged. However, the results support the possibility that SOD mimetics can be useful for the treatment of PH [Elmedal *et al.* 2004].

Erdosteine is metabolised in the liver and active metabolites with a thiol group are generated, which account for the free radical scavenging and the anti-oxidant properties of erdosteine. The hypobaric hypoxic rats showed reduced levels of lipid peroxidation, decreased inflammation and increased total antioxidant status.Erdostein also protected the pulmonary endothelium and reduced right ventricular hypertrophy [Uzun *et al.* 2006].

1.4.6. CoQ_{10} and curcumin in pulmonary hypertension

There were no preclinical reports of CoQ_{10} in PH in the literature to our knowledge, but there is an ongoing clinical trial with CoQ_{10} in PH [Clinicaltrials.gov]. There was one study carried out using curcumin in hypoxic hypercapnic rats. In their study curcumin decreased the mPAP, pulmonary vascular remodelling and inhibited the deposition of collagen I in pulmonary arterioles [Lin *et al.* 2006 (Abstract)].

1.5. Drug delivery and nanoparticles

Many research groups working on natural agents are trying to develop novel drug delivery systems to overcome the stability, bioavailability and clinical trial paradox [Belcaro *et al.* 2010]. Delivery of therapeutically active compounds to the body plays a key role in combating diseases and hence re-establishing the optimal physiological health state. The ideal drug delivery system should offer required pharmacokinetics (absorption, distribution, metabolism and elimination) and pharmacodynamics (action or effect of drugs) in a specified condition for which the system is intended. Active moiety is expected to be delivered by the delivery system at a rate and extent which is necessary to elicit the action at the site of action. Ideally, the system should be inert, safe and made up of a biocompatible material.

Therapeutic agents can be delivered via a number of routes including oral, transdermal and parenteral routes for systemic action. Among various routes of delivery, oral route is the preferred route of administration owing to its ease of administration, convenience of use by the patients on their own and absence of pain during administration leading to high patient acceptability and better compliance. Other advantage includes the availability of large surface area in the small intestine for absorption. One important consideration when developing therapeutic agents for delivery through the oral route is bioavailability. Bioavailability is defined as the rate and extent to which a drug/active ingredient is absorbed from its dosage form and becomes available at the specific site of action. Bioavailability refers to the actual measured active ingredient concentration in the blood which is the reason why intravenous delivery yields bioavailability of one hundred percent. For a therapeutic moiety to be orally active, it would have to be absorbed through the GI epithelium into the bloodstream. An orally active

agent needs to be sufficiently hydrophilic to dissolve in the GI fluids but at the same time should possess some degree of hydrophobicity to partition across the GI epithelial membrane. Active agents having oral bioavailability problems when loaded into nanoparticles resulted in increased bioavailability [Shaikh *et al.* 2009, Mittal *et al.* 2007, Italia *et al.* 2007], because of which nanoparticles are considered suitable for poorly bioavailable drugs through oral route.

The active agent is dissolved, adsorbed, attached or encapsulated in the polymeric matrix of nanometer size to give nanoparticles. Depending upon the method of preparation nanospheres or nanocapsules are obtained with different release and surface properties [Kumari *et al.* 2010]. The active ingredient is then slowly released from the polymeric coating over a period of time which in turn helps reduce dosing frequency [Mittal *et al.* 2007]. There are three proposed mechanisms of controlled release from the nanoparticles whch include the diffusion of the active ingredient from the nanoparticles, surface erosion of the matrix material and biodegradation of the nanoparticle matrix [Sahana *et al.* 2008, Mittal *et al.* 2009]. Nanotechnology as a delivery platform offers very promising applications through the oral route. In case of the active ingredients where efflux mechanisms play major role in poor oral bioavailability, these nanoparticles will help to improve their bioavailability by the unique absorption mechanism through the gut wall [Hussain *et al.* 2001].

1.5.1. Preparation techniques of nanoparticles

There are several methods available in the literature for the preparation of nanoparticles from preformed polymers. They include solvent evaporation, salting out, nanoprecipitation, dialysis and supercritical fluid technology. The most common method is the solvent evaporation which is

also known as emulsification-diffusion-evaporation (EDE) method [Rao and Geckeler 2011]. In this method, polymer solutions are prepared in volatile solvents and emulsions are formulated. Previously, dichloromethane and chloroform were widely used, but are now replaced with ethyl acetate which is safer. Sometimes all the actives are not soluble in ethyl acetate, in order to achieve maximum entrapment efficiency a co-solvent is sometimes used [Bala *et al.* 2005]. The emulsion is converted into a nanoparticle suspension after evaporation of the volatile solvent. The process variables are generally adjusted to obtain nanoparticles with maximum entrapment efficiency, optimum size and zeta potential.

In the conventional methods of nanoparticles preparation, two main strategies are being used for the formation of emulsions: the preparation of single-emulsion, oil-in-water (o/w) or double-emulsion, (water-in-oil)-in-water, (w/o)/w. These methods utilise high-speed homogenization or ultrasonication, followed by evaporation of the solvent, either by continuous magnetic stirring at room temperature or under reduced pressure. Afterwards, the solidified nanoparticles can be collected by ultracentrifugation and washed with distilled water to remove additives such as stabilisers.

1.5.2. Nanoparticle matrix material – Poly lactide-co-glycolic acid (PLGA)

Nanomaterials for drug delivery must meet several requirements, such as biocompatibility, compatibility with therapeutic agent, suitable biodegradation kinetics and mechanical properties, as well as flexibility for processing [Lu *et al.* 2009]. The advantages of using natural polymers include the low cost and biocompatibility. However, natural polymers may also be limited in their use due to the presence of extraneous contaminants, variability and low hydrophobicity. In contrast, synthetic polymers are more

reproducible and can be prepared with desired degradation rates, molecular weights and copolymer compositions. A wide variety of rigid polymers are available, which are suitable for encapsulation, delivery and controlled release of both low molecular mass active ingredients and biopolymer pharmaceuticals. Toxicity, irritancy, allergenicity, together biodegradability of the polymer are of primary concern.

The most commonly used polymer for delivery aspects is poly lactic-co-glycolic acid (PLGA) [Lu *et al.* 2009]. PLGA has been in focus in the search for appropriate matrices for nanoparticles due to its ease of preparation, commercial availability at a reasonable cost, versatility, biocompatibility and hydrolytic degradation into resorbable harmless products [Bala *et al.* 2004, Lu *et al.* 2009]. The forms of PLGA are usually identified by the monomers' ratio used e.g., PLGA 50:50 which is most frequently used in the nanomedicines, identifies a copolymer whose composition is 50% lactic acid and 50% glycolic acid. The popularity of PLGA is further explained by the fact that FDA has approved PLGA for a number of clinical applications [Bhardwaj *et al.* 2005].

1.5.3. Stabilisers

Stabilisers are used to prevent the aggregation of particles by conferring a surface charge. Normally higher the surface charge, greater is the stability. PVA is the most widely used stabiliser in the manufacture of PLGA nanoparticles. A fraction of PVA remains associated with the nanoparticles despite repeated washings because PVA forms an interconnected network with the polymer at the interface. This residual PVA on PLGA nanoparticles can be controlled by altering the PVA concentration or the type of organic solvent employed in the emulsion. Residual PVA can influence the physical properties of the particles (like size, zeta potential, polydispersity index, surface hydrophobicity), entrapment efficiency of the

active ingredient, cellular uptake and release. Hence residual PVA and factors influencing it can be used as formulation parameters to alter the properties or application of nanoparticles [Sahoo *et al.* 2002]. PVA with low degree of hydrolysis gives a higher yield, uniform size distribution and excellent redispersibility. Particle characteristics depend more on the degree of hydrolysis than on the degree of polymerization [Murakami *et al.* 1997].

Didodecyl dimethyl ammonium bromide (DMAB), a double-tail cationic stabiliser was found to form smaller size particles than routinely used stabiliser PVA. The extensive investigations demonstrate DMAB as a potential stabiliser generating stable nanoparticles in the range of 100 nm in size irrespective of the drug and polymer characteristics used [Grama *et al.* 2011]. The quaternary nature of DMAB raises safety concerns. However, at concentrations lower than 33 μM, which is the concentration found to be associated with the particles, DMAB was found to be non-toxic [Bhardwaj *et al.* 2009].

1.5.4. Entrapment efficiency

The factors deciding the entrapment efficiency of the active agent in the nanoparticles are method of preparation, stabilisers, solvent, nature of the therapeutic agent and the polymer. Formulation variables can be modulated to increase the entrapment efficiency in nanoparticles [Govendere *et al.* 1999]. During the preparation of PLGA nanoparticles by the w/o/w double emulsion technique, active ingredient (bovine serum albumin) loading was improved by varying the concentration of the protein in the inner aqueous phase. Higher protein concentration reduced the encapsulation efficiency [Lamprecht *et al.* 2000]. The solubility of the active agent has important outcomes on the entrapment efficiency during the preparation of nanoparticles. Entrapment in the precipitating polymer is a

function of the active ingredient's solubility in polymer, organic phase, aqueous phase, interaction with the stabiliser and polymer. The solubility and stability of the active ingredient will influence the choice of the solvent system, the stabiliser and the process employed, all of which can in turn, affect the entrapment efficiency. It is difficult to predict the correct combination of the above because our understanding of this science is, as yet based on exploratory rather than logistical approaches [Bhardwaj *et al.* 2005].

1.5.5. Bioavailability enhancement by nanoparticles

Nano delivery system are known to increase the bioavailability by increasing the apparent solubility of the active ingredient, increasing the rate of mass transfer, increasing the retention time or increasing the absorption via direct uptake of the nanoparticle carrier [Acosta 2009]. In accordance with other reports from literature, previous results from our lab using EA [Bala *et al.* 2006a], ciclosporine [Italia *et al.* 2007], oestradiol [Mittal *et al.* 2007], CoQ_{10} [Ankola *et al.* 2007] and curcumin [Shaikh *et al.* 2009] in PLGA nanoparticles suggest that the nanoparticulate approach enhances the bioavailability. Particle size was found to strongly influence the bioavailability of oestradiol [Mittal *et al.* 2007]. It has been proved that the nanoparticles are better than their suspension counterparts or marketed formulations in treating disease conditions. At 3 times lower dose than simple suspension EA loaded nanoparticles were proven to reduce the oxidative damage caused in ciclosporine induced nephrotoxicity [Sonaje *et al.* 2007]. In another study, the ciclosporine nanoparticles were observed to be more bioavailable than the marketed formulation of the same [Italia *et al.* 2007]. The nanoparticulate approach for atorvastatin calcium was successful in normalizing the dyslipidaemia and able to reduce the adverse effects caused by atorvastatin calcium in high cholesterol diet fed rats [Meena *et al.* 2008].

1.6. Uptake of nanoparticles

Small intestine is the major site of nanoparticles' uptake. The inner surface of the small intestine is covered with small "finger-like" protuberances called villi. Each epithelial cell is covered with even smaller protuberances called microvilli that helps increase the surface area for absorption. A mucous layer of an anionic glycoprotein (mucin) typically covers the surface of the microvilli and represents a key factor in the uptake of nanoparticles [Lai *et al.* 2009]. Some researchers [Kreuter 1991; Desai *et al.* 1996, Hussain *et al.* 2001] strongly support the idea that direct nanoparticle uptake from intestines is a viable route for the delivery of active ingredients. These and other researchers have proposed three main routes of nanoparticle uptake: paracellular uptake, transcellular (transcytosis) uptake by enterocytes (which represent 90–95% of the epithelial cells of the intestine) and transcellular uptake by M (microfold) cells. The paracellular uptake (uptake through the interstitial space between epithelial cells) is considered to be the least effective of the three mechanisms because the space between cells ranges between 0.3 and 1 nm, which is too small for most nanoparticles to permeate. There are two methods of nanoparticles transcytosis, one is passive in which case the nanoparticles diffuse through the epithelial cell and the second method involves the presence of receptors on the surface of the cells that capture nanoparticles with specific surface chemistry. Although M cells are the ideal portal for micro- and nanoparticles delivery, the problem with this approach is that these M cells represent typically less than 1% of the total intestinal area, which makes the selective delivery to these sites more difficult [Hussain *et al.* 2001].

Nanopaticles are capable of gaining intracellular access in all cell lines investigated in various studies [Panyam *et al.* 2003, Prabha *et al.* 2002]. The rate and the extent to which nanoparticles were taken up was different with

the type of cells employed. In a study 3 different kinds of epithelial cells were used to study the uptake of fluorescent dye rhodamine entrapped PLGA nanoparticles viz. (1) opossum kidney (OK) cells – model of renal proximal tubule, (2) Caco-2 cells – model of the gut epithelium, and (3) human bronchial epithelial (HBE) cells – model of the respiratory airway. Cross-sectional slices of the images confirmed that nanoparticles were indeed inside the cells and not simply adsorbed to the outer surface. OK cells took up the PLGA nanoparticles quickly, as early as 30 min post addition, nanoparticles were found within the cells and it appears that a saturation limit was reached at about 4 h. In both the Caco-2 and HBE cells, the rate of uptake was considerably slower than in OK cells. Saturation limit was not observed in HBE cells within the 24 h period. To determine whether the uptake mechanism of NP entry into epithelial cells was mediated by endocytosis, cells were co-incubated with nanoparticles and maintained at 4°C. Endocytosis, an energy dependent process, is blocked at low temperatures. It was observed that nanoparticle uptake was significantly decreased at 4°C suggesting that indeed uptake is mediated by endocytosis [Cartiera *et al.* 2009]. Other work using nanoparticles in Caco-2 cells also demonstrates that endocytosis is the mechanism of uptake within the cells [Desai *et al.* 1997]. Analysis of immunofluorescently labelled compartments in OK and HBE cells showed that internalized nanoparticles colocalized with markers of early endosomes and the Golgi complex. Given that nanoparticles are not highly colocalized with late endosomes or lysosomes relative to the other compartments; it can be inferred that these nanoparticles may largely escape endo-lysosomal degradation [Cartiera *et al.* 2009].

The uptake studies so far in the literature were based on fluorescent techniques, transmission electron microscopy, radio-labelling or gamma

scintigraphy [Bhardwaj *et al.* 2005]. It is very important to know the mechanism of absorption, distribution profiles of nanoparticles elaborately to make the most use of nanoparticles. It is possible to modify the characteristics of nanoparticles accordingly to the need of the disease if the absorption mechanisms and distribution profiles of the nanoparticles are known. There were no techniques reported to track the nanoparticles using simple microscopy or routine histological stains. This led us to ask if β-galactosidase (β-gal) could be used as a marker for nanoparticles in tissue sections. The enzyme β-gal is E. coli-derived and has been widely used as a reporter gene product and marker enzyme. Commonly used substrate is 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) [Hung *et al.* 2001], and it gives a colour reaction product that is readily visualized by light microscopy [Romanos *et al.* 1992; Lampson *et al.* 1993].

1.7. Aims

Selection of the active ingredients for nanoparticulate delivery approach is crucial to the success of the technology. The selection criteria includes, molecules that are difficult to be delivered by conventional means due to poor biopharmaceutical and pharmacokinetic properties which include poor solubility and permeability, narrow therapeutic index, high toxicity, high target specificity, P-glycoprotein efflux, etc., along with intellectual property rights [Grama *et al.* 2011].

The role of oxidative stress in pathogenesis or disease progression of atherosclerosis, diabetes and pulmonary hypertension is clear and the diseases highlighted in blue are dealt with in this thesis [Fig. 1.3]. The objective of this thesis is therefore to establish the therapeutic efficacy of NanoCAPs, CoQ_{10} and curcumin in the diseases highlighted in Fig. 1.3 and to

study the uptake of β-gal loaded nanoparticles via intestine. The objectives will be achieved by the following specific aims:

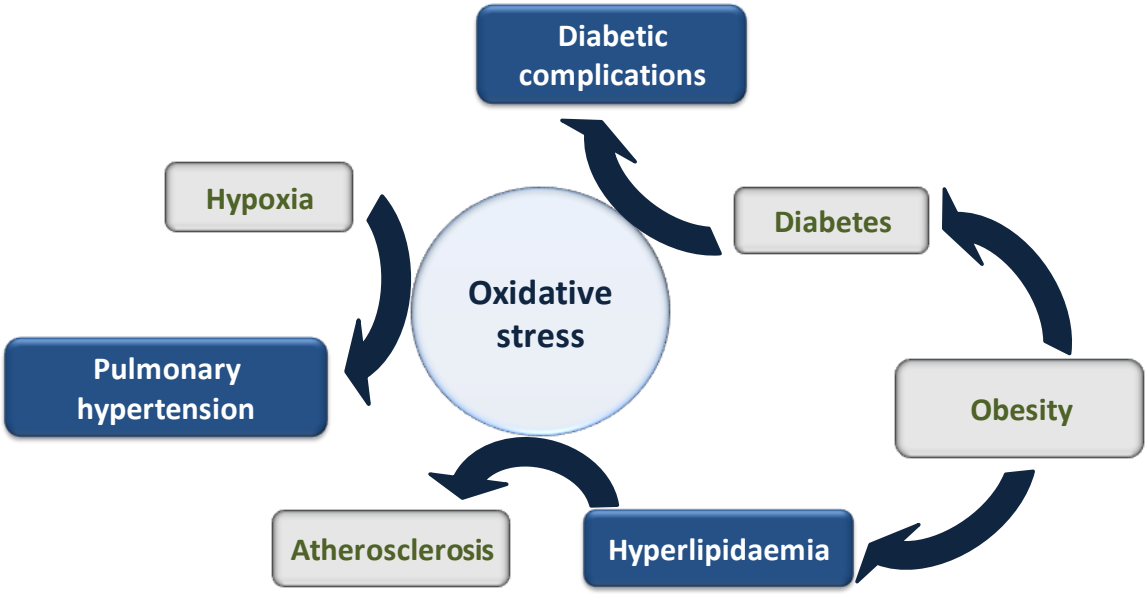


Figure 1.3. Figure depicting oxidative stress mediated diseases.

- Preparation and characterization of NanoCAPs, CoQ_{10} and curcumin nanoparticles.
- Evaluation of the antihyperlipidaemic activity of NanoCAPs in high fat diet fed rats
- Evaluation of the ability of CoQ_{10} and Curcumin nanoparticles to prevent the development of diabetic complications in STZ induced diabetes in rats
- Evaluation of the beneficial properties of CoQ_{10} and curcumin nanoparticles in chronic hypoxia induced pulmonary hypertension in rats
- Develop, characterise and utilise β-gal loaded nanoparticles for understanding particle absorption in rodents.

2. FORMULATION OF NANOPARTICLES

2.1. Introduction

Nanoparticles are explored for oral delivery of many challenging molecules which are limited in their use because of their poor biopharmaceutical and pharmacokinetic properties. Entrapment of active ingredients in polymeric nanoparticles results in improved stability [Kwon *et al.* 2002], uptake [Bala *et al.* 2006b] and distribution profiles [Mittal *et al.* 2007]. Nanoparticles upon peroral administration are believed to be taken up intact [Hussain *et al.* 2001] and circulate for extended periods in the blood releasing the entrapped agent in a sustained fashion resulting in dose reduction thereby reducing adverse effects [Sonaje *et al.* 2007, Italia *et al.* 2007].

In vitro activities of natural agents like EA, CoQ_{10} and curcumin reveal very strong antioxidant and anti-inflammatory potential [Hayes *et al.* 2011, Kunitomo *et al.* 2008, Ak and Gulcin 2008], however, this does not reflect *in vivo* due to their poor biopharmaceutical properties [Bala *et al.* 2006, Bhagavan and Chopra 2006, Jain *et al.* 2009]. Poor *in vivo* performace is because of their poor solubility and permeability across the intestinal wall and extensive biotransformation when delivered through the oral route [Pan *et al.* 1999,Ratnam *et al.* 2006, Bank *et al.* 2011,]. The nanoparticulate approach for these molecules has proven to be successful in increasing the deliverability of these active ingredients thereby bioavailability/efficacy [Sonaje *et al.* 2007, Zmitek *et al.* 2008a, Shaikh *et al.* 2009].

Emulsion-diffusion-evaporation method is suitable for a range of molecules with diverse physicochemical properties, however suitable stabiliser/solvent screening is necessary [Bhardwaj *et al.* 2005]. If the active ingredient is lipophilic/hydrophobic oil in water (o/w) emulsification method

is used. In some cases when the active ingredient lacks solubility in the oil phase, a co-solvent is used. A stabiliser must be used to stabilise the emulsion during the solvent evaporation process. While manufacturing the nanoparticles entrapping proteins or enzymes it is important to get the nanoparticles retaining the active group through the conditions of the nanoparticles manufacture [Dziubla *et al.* 2005]. The method has to be modified according to the enzyme. The stability of the enzyme is very crucial in the development of nanoparticles for enzymes as the stability is limited over a pH range and temperature. Co-encapsulation is another approach adapted in the present research program which is an interesting approach to obtain uniform particles containing two or more agents with same size distribution and surface characteristics within a single formulation. When two or more ingredients are added as actives in the preparation of nanoparticles the solubility of all the actives must be taken into consideration to achieve maximum entrapment efficiencies.

Particulate adhesion and uptake in the GI tract was shown to be involved in the efficacy of oral drug delivery and is affected by the surface properties and the particle size [Tobio *et al.* 2000, Li *et al.* 2007]. The preferable particles are small and hydrophobic particles as they have higher uptake efficiency [Prabha *et al.* 2002]. Mucus acts as a sticky net in the GI and clears the nanoparticles very rapidly. Mucus penetrating nanoparticles are prepared with a view to increase the retention time of nanoparticles and to enhance the sustained release of the loaded agent [Lai *et al.* 2009]. A dense PEG coating, as characterized by a near-neutral surface charge and negligible protein adsorption, improved the transport of nanoparticles by up to 3 orders of magnitude for particles in the range of 100–500 nm in size. The greatly improved nanoparticle transport rates upon PEGylation correlated

with a sharp decrease in the fraction of immobile particles, less than 0.5% of 200 and 500 nm PEGylated particles were immobilized by mucus compared to 32 and 45% for uncoated 200 and 500 nm particles, respectively [Lai *et al.* 2007]. Although there has been a major literature support suggesting the benefits of nanoparticles through the oral route, the mechanisms of absorption are still not completely understood. This area still remains open for studies explaining the uptake and fate of nanoparticles after oral ingestion.

Very recently, we have reported a process to make co-encapsulated nanoparticles (NanoCAPs) of EA and CoQ_{10} [Ratnam *et al.* 2008]. The developed NanoCAPS were better than the respective individual agents or their combination administered as plain suspension as well as demonstrated synergistic effects in ameolerating STZ induced inflammation in rats. This chapter describes the preparation and characterization of NanoCAPs of EA & CoQ_{10}, CoQ_{10} encapsulating nanoparticles, curcumin encapsulating nanoparticles and β-gal encapsulating nanoparticles. The preparation and characterisation of NanoCAPs of EA and CoQ_{10} was carried out at National Institute of Pharamaceutical Education and Research (NIPER), S.A.S. Nagar, India.

2.2. Materials and methods

2.2.1. Materials

PLGA (Resomer R503H; M.W 35-40 kDa) was purchased from Boehringer Ingelheim, Ingelheim, Germany. EA, β-galactosidase (1 mg equivalent to 250 IU), polyvinyl alcohol (PVA) (Mol. Wt. 30,000–70,000), didodecyl dimethyl ammonium bromide (DMAB), ethyl acetate, propylene glycol, potassium dihydrogen orthophosphate, sodium acetate, magnesium chloride, ortho-nitrophenyl-β-galactoside (ONPG), and 5-bromo-4-chloro-3-

indolyl-β-D-galactopyranoside (X-gal) were purchased from Sigma. HPLC grade methanol, ethanol and acetonitrile were procured from J.T. Baker, USA. Curcumin and CoQ_{10} were gift samples from Indsaff Punjab, India and Tischon Corp, Westbury, NY respectively. Poly (ethylene glycol)-400 (PEG-400) was purchased from S.d. fine, India.

2.2.2. Preparation of nanoparticles

2.2.2.1. Nano-co-encapsulated antioxidant particles (NanoCAPs) of EA and CoQ_{10}

The NanoCAPs of EA and CoQ_{10} were prepared using an emulsion technique incorporating both the compounds in a single formulation. The method goes as follows: 50 mg PLGA and 5 mg CoQ_{10} (10% w/w of polymer) were dissolved in 2.5 ml ethyl acetate and allowed to stir at 1000 rpm for 30 min using a magnetic stirrer to obtain a homogeneous solution. Five mg EA (10% w/w of polymer) was dissolved in 0.6 ml PEG-400 and stirred for 30 min. These two solutions were mixed and stirred for 1 min to form the organic phase. The organic phase containing the active ingredient and polymer was then added in a drop wise manner to 5 ml of 1% aqueous PVA solution while homogenisation. Homogenisation using a tissue homogeniser was continued for 5 min at 15,000 rpm, followed by addition of 20 ml water to facilitate diffusion of ethyl acetate that was subsequently evaporated by overnight stirring. The preparation was then centrifuged at 20,000g for 5 min to separate the supernatant containing free actives and unbound stabiliser. The pellet was redispersed in the required amount of water and characterised.

2.2.2.2. Coenzyme Q_{10} and curcumin nanoparticles

The CoQ_{10} and curcumin loaded nanoparticles were prepared by a modified emulsion-diffusion-evaporation method previously developed in

our laboratory [Hariharan *et al.* 2006]. Briefly, 50 mg PLGA and 10 mg CoQ_{10} (20% w/w of polymer) or 7.5 mg curcumin (15% w/w of polymer) were dissolved in 2.5 ml of ethyl acetate and allowed to stir at 1000 rpm for 30 min to obtain a homogeneous solution. The organic phase containing the active ingredient and polymer was then added in a drop wise manner to 5 ml of 1% aqueous stabiliser solution (DMAB for CoQ_{10} and PVA for curcumin) while homogenisation. Homogenisation was continued for 5 min at 15,000 rpm. After homogenisation the emulsion was transferred to 20 ml water to facilitate diffusion and was stirred overnight to ensure the complete evaporation of the organic solvent. After the evaporation was complete, the nanoparticles solution was centrifuged at 15,000g for 15 min to separate any unbound stabiliser in the solution. The supernatant was separated and the pellet was redispersed in the required amount of water.

2.2.2.3. β-galactosidase nanoparticles

β-gal loaded nanoparticles were prepared following the emulsion-diffusion-evaporation method with modifications [Bala *et al.* 2005]. β-gal was dispersed in propylene glycol, which is used with a view to stabilize the enzyme β-gal and dissolve the salts and sugars associated with the freeze dried product. The aqueous phase is generally water containing a stabiliser. In this experiment pH 6.0 buffer with 100 mM concentrations of sodium acetate and magnesium chloride were used. The buffer was prepared by dissolving the required amounts of sodium acetate and magnesium chloride (100 mM concentrations of both the components) in water. Then the pH was adjusted to 6.0 with 1M glacial acetic acid. The prepared buffer was used to solubilise the stabiliser PVA. 2 mg β-gal, approximately 500 IU, was added to 0.3 ml propylene glycol and stirred for 5 min. This solution was added to 2 ml ethyl acetate solution containing 50 mg PLGA. This organic phase was

stirred using a magnetic stirrer for at least 15 min. In a separate vial 50 mg PVA was dissolved in 5 ml buffer solution. While homogenisation, using tissue homogeniser, the solution containing β-gal and polymer was added in a drop wise manner to PVA buffer solution. Homogenisation was carried out at 15,000 rpm for 2 min. Later on, the formed emulsion was added to 20 ml buffer solution on the magnetic stirrer, stirring at 800 rpm. To facilitate complete diffusion followed by evaporation of the organic solvent stirring was continued for at least 15 h. Then, the nanoparticles were centrifuged at 15,000 g for 5 min and the pellet was washed with the buffer solution and the supernatant, which contains unbound stabiliser, was discarded. The pellet was redispersed in required amount of buffer solution for characterization.

2.2.3. Characterization of nanoparticles

2.2.3.1. Size and zeta potential

The nanoparticles were characterized using a zetasizer (Nano ZS, Malvern Instruments, Malvern, UK) for size (average of 5 measurements for one batch) and zeta potential (average of 20 measurements for a batch). The average of three batch measurements was expressed as mean ± standard deviation. The medium for the size and zeta potential measurements was water in case of antioxidant nanoparticles and buffer in case of β-gal nanoparticles.

2.2.3.2. Surface characterization

2.2.3.2.1. Atomic force microscopy (AFM)

The surface morphology (of NanoCAPS) was analysed using AFM (Veeco Bioscope II) that was attached to a Nikon eclipse TE 2000-S microscope. The system was run by Nanoscope software. The nanoparticles suspension was placed on a silicon wafer with the help of a micro pipette and allowed to dry in air. The microscope was vibration damped and

measurements were made using commercial pyramidal Si_3N_4 tip (Veeco's CA, USA). The cantilever used for scanning has a length 325 µm and width 26 µm with a nominal force constant 0.1 N/m. For all the nanoparticles, the scan size was 5 µm with scan rate of 0.996 Hz. The images were height images obtained in contact mode.

2.2.3.2.2. Transmission electron microscopy (TEM)

The morphology of the prepared CoQ_{10}, curcumin and β-gal nanoparticles was analysed using TEM (LEO 912, Cambridge, UK). Carbon-coated 200 mesh copper grids were glow discharged and specimens in distilled water were dried down to a thin layer onto the hydrophilic support film. Twenty µl of 1% aqueous methylamine vanadate (Nanovan, Nanoprobes, NY, US) stain was applied and the mixture dried down immediately with filter paper to remove excess liquid. The dried specimens were imaged with a LEO 912 energy filtering transmission electron microscope at 120 kV. Contrast enhanced, zero-loss energy filtered digital images were recorded with a 14 bit/2K Proscan CCD camera.

2.2.4. Entrapment efficiency

2.2.4.1. NanoCAPs of EA and CoQ_{10}

The actual amount of active encapsulated within the nanoparticles from the total active initially used is represented as entrapment efficiency (EE). To estimate the EE, nanoparticles were centrifuged at 20,000 g for 5 min followed by two washings with 10 ml water to remove free active and unbound stabiliser. The pellet was extracted for EA and CoQ_{10} using methanol by probe sonication (UP200S Ultrasonic processor, Germany) for 2 min at maximum intensity followed by quantification using HPLC. Simultaneous analysis of EA and CoQ_{10} was performed using Supelco Discovery® HS PEG column, 25 cm × 4.6 mm, 5 µm (Supelco, Bellefonte, PA,

USA) maintained at 40°C. Elution of both the compounds was achieved using gradient mobile phase (acetonitrile–ethanol–5 mM potassium dihydrogen orthophosphate buffer (pH 2.5) (70:15:15, v/v/v) for 15 min except for 2.5–7 min where acetonitrile–ethanol (30:70, v/v) was used instead. The EE was calculated by analyzing the amount of active ingredient present in the nanoparticles directly by the following formula.

$$EE\ (\%) = \frac{(\text{Amount of entrapped active agent}) \times 100}{\text{Amount of active agent initially added}}$$

2.2.4.2. Coenzyme Q_{10} and curcumin nanoparticles

CoQ_{10} and curcumin nanoparticles EE were calculated by indirect method, i.e., measuring the amount of free active in the supernatant and subtracting it from the initial amount loaded. Entrapment efficiency was determined by centrifuging the active ingredient loaded nanoparticles at 15,000g for 15 min and separating the supernatant. The supernatant was analysed for free CoQ_{10} and curcumin. CoQ_{10} was analysed using HPLC. Separation was achieved using a reversed phase C18 column fitted with guard column. A mixture of 9:1 of ethanol and methanol was used as the mobile phase at a flow rate of 1 ml/min with UV detection at 275 nm. Curcumin was analysed using a fluorescence spectrophotometer at an excitation wavelength of 420 nm and emission wavelength of 530 nm. The entrapment efficiency was calculated using the following formula.

$$EE\ (\%) = \frac{(\text{Initial amount} - \text{Amount in the supernatant}) \times 100}{\text{Amount of active agent initially added}}$$

2.2.4.3. β-gal entrapment in nanoparticles

β-gal entrapment efficiency was calculated by measuring the amount of active enzyme indirectly. The indirect method involves quantification of unloaded enzyme (IU) and then subtracting from the total enzyme (IU) initially loaded in the preparation process to get the amount of active β-gal present in the nanoparticles. The β-gal activity assay was carried out using ONPG as the enzyme substrate. The nanoparticles were washed for the excess surfactant and unloaded enzyme by centrifuging at 10,000g for 10 min and then the supernatant was separated out. The amount of the enzyme (IU) present in the supernatant was calculated by the following method after diluting it 10 times [Bahl *et al.* 1969]. 0.5 ml of 2 mM ONPG was added to a vial, followed by 0.3 ml deionised water and 0.2 ml enzyme solution. The contents were mixed thoroughly using a vortex and then incubated for 10 min at 37°C. After 10 min, 4 ml of 1M sodium carbonate solution was added to stop the enzyme reaction. Then the UV absorbance at 405 nm was recorded for the formed O-nitro phenol. Blank absorbance at 405 nm was recorded from the mixture containing the substrate (ONPG) and water which was incubated for 10 min and then added 4 ml sodium carbonate followed by 0.2 ml enzyme solution. Enzyme activity was calculated using the following formula.

Units/ml enzyme = (A_{405} Test $-A_{405}$ Blank) (5) (Df) / (10) (4.6) (0.2)

Where, 5= total volume of the assay (ml)

Df = dilution factor

10 = time of assay (in minutes)

4.6 = milli molar extinction co-efficient of O-nitro phenol at 405 nm

0.2 volume of the enzyme used (ml)

After determining the active enzyme present in the supernatant the β-gal entrapment efficiency was calculated using the following formula. The enzyme loading efficiency was calculated by the amount of active enzyme but not by the enzyme content which might also include inactive enzyme.

β-gal EE (%) = (Initial β-gal – β-gal in supernatant) (100) / (Initial β-gal)

2.3. Results

NanoCAPs of EA and CoQ_{10} were prepared successfully following the emulsion diffusion evaporation method. The Z-average particle size obtained was 259±1 nm and zeta potential of −17.1±2.6 mV [Table 2.1 & Fig. 2.1A].From the AFM micrograph it can be seen that the particles are spherical in shape [Fig 2.2.]. The entrapment efficiency at 10% initial loading for the NanoCAPs is 70.1±1.4 and 72.4±0.2% for EA and CoQ_{10}, respectively.

CoQ_{10} and curcumin loaded nanoparticles have been prepared successfully. The CoQ_{10} and curcumin loaded particle Z- average sizes were 115±12 nm and 237±6 nm respectively [Fig. 2.1B&C]. The entrapment efficiencies of nanoparticles of CoQ_{10} and curcumin nanoparticles were determined to be 70±3% and 66±3% respectively with 20% w/w and 15% w/w initial loading of the polymer PLGA. The TEM images indicated spherical shape of nanoparticles [Fig. 2.3A&B].

On the other hand, the size of the β-gal entrapped nanoparticles was 307±18 nm [Fig.2.1D & Fig. 2.3C] with low polydispersity index (PDI) of 0.134 [Table 2.1]. β-gal loading efficiency was calculated to be around 47.9±3.4% of the initial loading.

The TEM micrographs show differences in the sizes of the nanoparticles stabilised with DMAB and PVA [Fig. 2.3]. DMAB yielded positively charged nanoparticles and PVA yielded negatively charged nanoparticles except for β-gal loaded nanoparticles [Table 2.1].

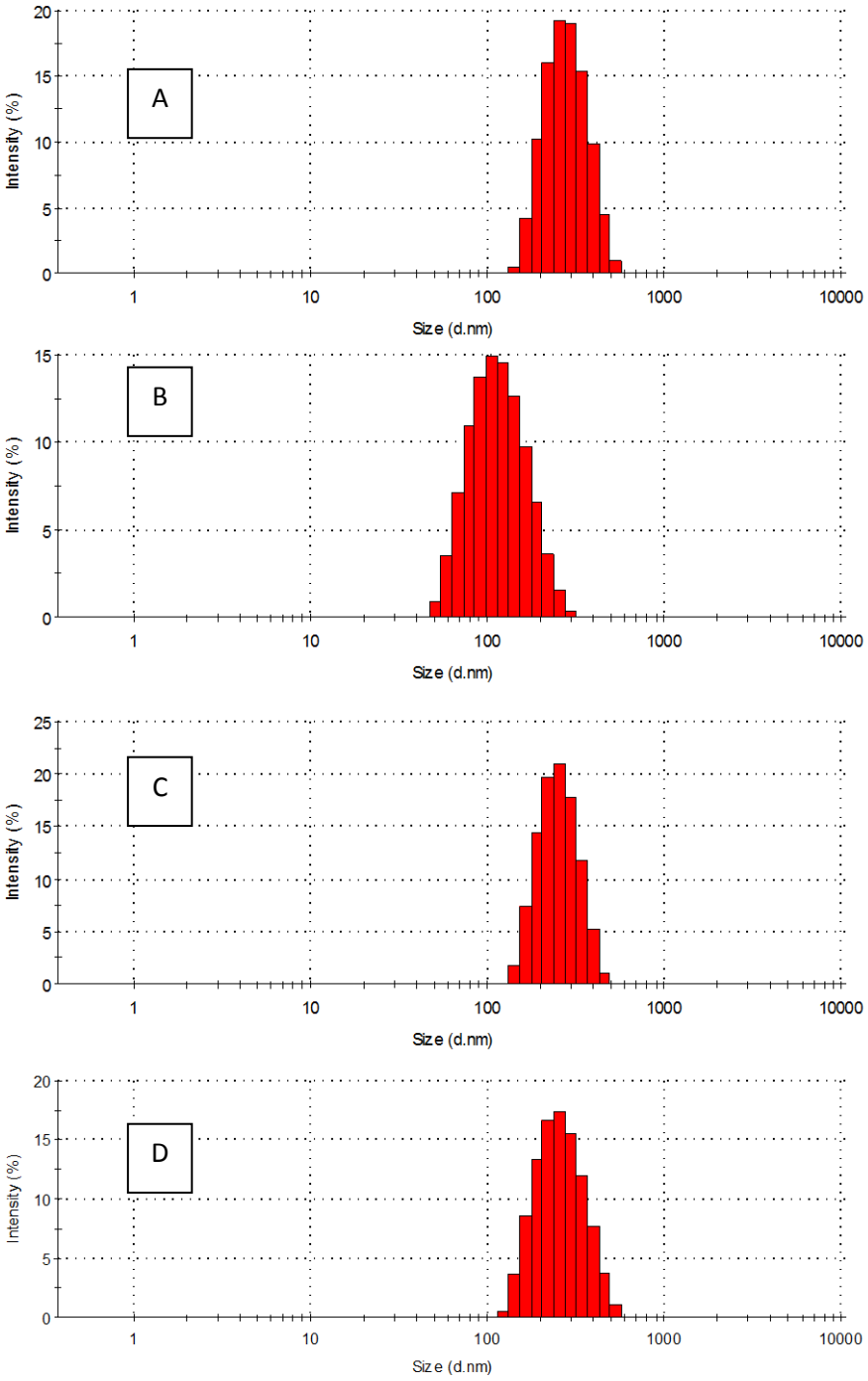


Fig.2.1. Particle size distribution of NanoCAPs (A), coenzyme Q_{10} (B), curcumin (C) and β-gal loaded nanoparticles (D).

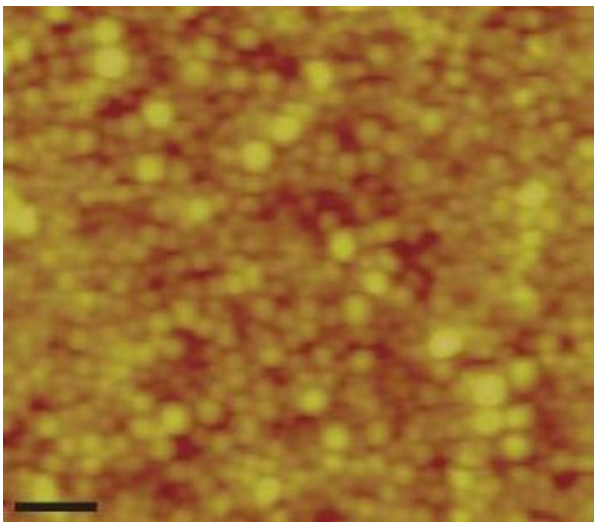


Fig. 2.2. AFM micrograph of the NanoCAPs. The scale bar indicates 600 nm. The image is height image showing similar sizes at similar height indicated by same colour.

Table 2.1. Nanoparticle characteristics

Nanoparticles	Size (nm)	Zeta potential* (mV)	PDI	Loading efficiency (%)
NanoCAPs	259±1	-17.1 ±2.6	0.269±0.005	70.1 ± 1.4 (EA) 72.4 ± 0.2 (CoQ_{10})
CoQ_{10}	115±12	75.4 ± 9.5	0.186±0.003	70.3±3.4%
Curcumin	237±6	-10.8 ± 1.9	0.172±0.098	66.2±2.6%
β-gal	307±18	6.5 ± 2.7	0.134±0.085	47.9±3.4◊

* The pH was at around 6 while measuring the zeta potential values

◊ Loading efficiency is the percentage IU of enzyme present after the preparation process; n=3; The continuous phase was water in all the measurements.

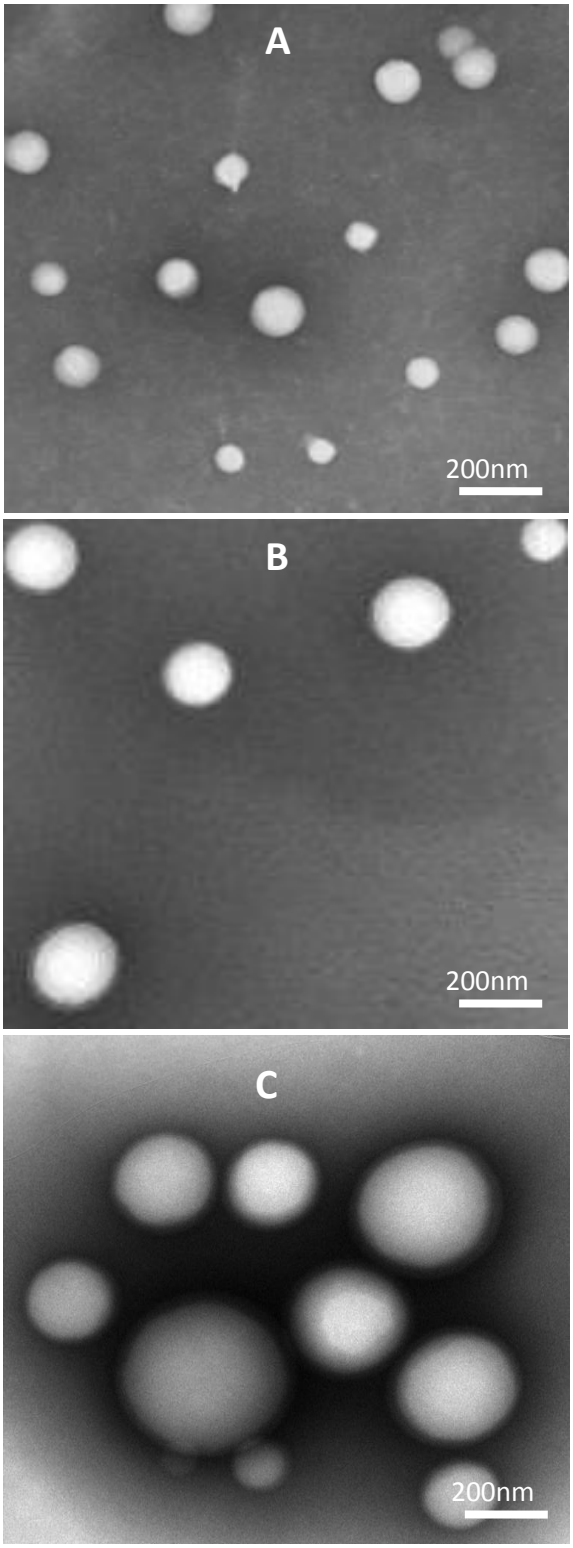


Fig. 2.3. Transmission electron micrographs of CoQ_{10} (A), curcumin (B) and β-galactosidase (C) loaded nanoparticles showing spherical nanoparticles.

2.4. Discussion

2.4.1. NanoCAPs of ellagic acid (EA) and CoQ_{10}

EA and CoQ_{10} have been successfully co-encapsulated into PLGA nanoparticles. The choice of a particular method for encapsulation of a substance in a colloidal carrier is most commonly determined by the solubility characteristics of the active ingredient as well as the polymer. Pharmaceutical compounds are soluble in either aqueous or non-aqueous solvents, which facilitate incorporation of these compounds into the nanoparticles when the emulsification technique was adopted. The solubility of EA is very poor in routinely used organic solvents like ethyl acetate or dichloromethane for preparation of nanoparticles, therefore a co-solvent PEG-400 was used to solubilize EA [Bala *et al.* 2005]. CoQ_{10} is soluble in ethyl acetate that is used to solubilize the PLGA for nanoparticle preparation. The previously reported method involves 3 h primary emulsification step where PEG-400 containing EA was mixed with polymeric solution followed by addition of this mixture to the stabiliser phase and stirred for 3 h [Bala *et al.* 2005]. Though PEG-400 could take EA to 5-15% of the initial weight of polymer the encapsulation decreased with increase in active ingredient load [Bala *et al.* 2006], which could be due to the phase separation and precipitation of active ingredient during 3 h emulsification step. The exclusion of 3 h emulsification in the current process resulted in significant improvements in entrapment efficiency, with the current process the entrapment efficiency of EA was increased to 10% w/w of polymer and at the same time allowed to co-encapsulate CoQ_{10} at 10% w/w of polymer resulting in ~70% encapsulation of both EA and CoQ_{10}.

2.4.2. Coenzyme Q_{10} and curcumin nanoparticles

CoQ_{10} nanoparticles were smaller than curcumin nanoparticles. The differences in nanoparticles sizes can be attributed to the characteristics of the stabilisers used. The stabiliser DMAB results in smaller sized particles in comparison to PVA which results in slightly larger particles. However, in case of curcumin it was not possible to formulate the particles with DMAB, because of the precipitation of curcumin during nanoparticle preparation process in the presence of DMAB. The initial loading of CoQ_{10} was 20% and curcumin was 15% w/w of polymer. Curcumin loading more than 15% resulted in larger size distributions. Other reports in the literature also state the solubility problem encountered when higher loading was attempted. A study states that loading more than 17% of curcumin was not possible due to the poor solubility of curcumin while preparing curcumin loaded poly (ε-caprolactone) nanofibers [Merrel *et al.* 2009]. The initial loading of the curcumin was limited by the solubility of curcumin in ethyl acetate which was used as organic phase in nanoparticle preparation process. The differences in entrapment efficiencies of CoQ_{10} and curcumin nanoparticles can be attributed to the properties of stabilisers used and the antioxidant physicochemical properties *per se*. CoQ_{10} is highly soluble but curcumin has limited solubility in ethyl acetate . The solubilities of these agents might have also played a role in varying entrapment efficiencies.

The stabilzer is believed to form a coat around the particle [Sahoo *et al.* 2001], thus has major effect on the surface charge. Zeta potential of CoQ_{10} particles was positive and curcumin particles was negative depending on the stabiliser characteristics. DMAB is a quaternary ammonium salt having cationic nature. Owing its cationic nature it gives a high positive charge to the nanoparticles [Bhardwaj *et al.* 2009]. PVA on the otherhand is a polymer without any charge and it gives the nanoparticles a slight negative charge.

2.4.3. β-galactosidase loaded nanoparticles

A modified emulsion-diffusion-evaporation process was optimized to obtain β-gal loaded nanoparticles and most importantly to get active enzyme loaded on to the nanoparticles [Bala *et al.* 2006]. Several modifications were carried out in the preparation process like controlling the temperature while emulsification process at 4°C, reducing the homogenisation speed from 15,000 to 5,000 rpm and using different types of surfactants like DMAB and PVA. However, none of these modifications could preserve the enzyme activity.

The stability of β-gal at 37°C decreases markedly with rising pH in the range 5.9-8.0 [Heyworth *et al.* 1981]. Furthermore, in the literature microspheres of β-gal have been reported to show different antigenicity and immunogenicity with different solvents employed in the formulation [Stivaktakis *et al.* 2004]. Thus, it can be implied that the pH, ionic strength and solvents have definite roles in the stabilization of β-gal. Therefore, considering these facts, an aqueous solution at pH 6 with 100 mM ionic strength was used instead of water in various steps of the preparation process of nanoparticles. This strategy resulted in nanoparticles without complete loss of the enzyme activity, which was confirmed by analysing the enzyme present in the supernatant. The literature suggests the use of double emulsion process for preparation of most of the enzyme loaded nanoparticles [Dziubla *et al.* 2005, Dziubla *et al.* 2008]. However, in single emulsification process, with the use of a co-solvent, suitable conditions like pH and ionic strength for a specific enzyme will prevent the loss of enzyme activity. The present method provides extended conditions for the enzyme to remain stable. In this way, a new approach to encapsulate β-gal has been developed, which in general can be extended to other sensitive proteins and peptides.

The entrapment efficiency was calculated using indirect method, by measuring the enzyme activity in the supernatant obtained after centrifuging the nanoparticles preparation at 10,000g for 10 min which contains unentrapped enzyme. Enzyme entrapment efficiency by the optimised preparation method resulted in ~48% of the initial loading.

2.5. Conclusion

NanoCAPs were prepared using a modified emulsion-diffusion evaporation method. PEG 400, co-solvent used in the preparation process facilitated appreciable entrapment of EA in the particles. CoQ_{10} and curcumin loaded nanoparticles were prepared following the methods already developed in our laboratory. In case of curcumin the loading was limited to 15% because of the limited solubility of curcumin in ethyl acetate. The process to encapsulate β-gal into the nanoparticles was successfully developed by optimising the surfactant combination, pH and ionic strength. The activity of enzyme β-gal was retained during the preparation process with propylene glycol as a co-solvent and pH 6.0 buffer with 100 mM concentrations of sodium acetate and magnesium chloride. In conclusion, EDE method can be used for small molecules as well as macromolecules. Specific modifications are needed in the selection of co-solvent, and stabilisers to achieve the optimum size and particle characteristics for antioxidant molecules. For enzyme encapsulation, stabiliser, co-solvent, pH, and ionic strength can be varied to get the active β-gal encapsulated.

3. NANO-CO-ENCAPSULATED ANTIOXIDANT PARTICLES (NANOCAPS) OF ELLAGIC ACID AND COENZYME Q_{10} IN HYPERLIPIDAEMIC RATS

3.1. Introduction

There is substantial evidence to suggest that obesity can be acquired by food habits and life style changes. Obesity is characterised by circulatory lipid levels and high levels of fat deposition in the body. High levels of cholesterol pose a major risk factor for many of the atherosclerotic diseases [reviewed in Jain *et al.* 2007]. Depending upon the risk assessment for cardiovascular diseases reducing the cholesterol levels as a part of therapy is always considered to reduce the risk of cardiovascular diseases. LDL-C as low as 25–60 mg/dL is physiologically sufficient [Brown and Goldstein 1986]. Apart from cholesterol, triglyceride levels were also linked independently to heart diseases [Austin *et al.* 1998]. Control of lipid levels is also helpful in diabetic dyslipidemia and metabolic syndrome. Therapeutic life style changes with combination of antihyperlipidaemic drugs are applied for the management of hyperlipidaemia. Although the available drugs treat hyperlipidaemia efficiently they are known to be associated with some serious adverse effects in some cases [Thompson *et al.* 2003].

Many plant extracts and natural compounds have been shown to be active in the management of hyperlipidaemia. However, they were never the mainline therapies, because of the poor characterisation of the extracts and poor solubility leading to poor bioavailability [Marinangeli and Jones 2010]. EA is a polyphenol mainly found in pomegranates and strawberries and has shown to reduce the elevated lipid levels in hyperlipidaemic rabbits. A study conducted in rabbits, which were fed cholesterol rich food, suggests that EA (1% in diet) has beneficial effects in alleviating the TC, TG and LDL-C levels

which were altered in cholesterol fed rats. TC, TG, and LDL-C levels in cholesterol fed group were 23.9±2.3 mmol/L, 4.2±0.6 mmol/L and 20.1±1.2 mmol/L respectively, while in the normal diet group the levels were 2.3±0.2 mmol/L, 0.9±0.1 mmol/L and 1.3±0.1 mmol/L respectively. The EA treated group had significantly reduced lipid levels in comparison to the cholesterol fed group where the TC, TG and LDL-C levels were found to be 10.3±2.1 mmol/L, 1.7±0.2 mmol/L and 7.9±2.1 mmol/L respectively. EA almost brought the TG levels to normal levels and TC levels although lowered significantly still higher than the normal levels in rabbits fed atherogenic diet. EA has also reduced the aortic lipid lesions in the rabbits fed atherogenic diet and the effect is suggested to be because of the antioxidative properties of EA [Yu *et al.* 2005].

CoQ_{10} is a lipid-soluble antioxidant synthesised in living organisms which has a unique function in mitochondria, as it transfers electrons from primary substrates to the oxidase system and at the same time it transports protons from the mitochondrial matrix to the intermembrane space. Because of the presence of a high percentage of ubiquinol (reduced form of CoQ_{10}) in plasma, there has been interest in the function of CoQ_{10} both as an endogenous antioxidant and as a therapeutic agent. CoQ_{10} had alleviated the lipid levels in fructose fed rats. Fructose (10% in drinking water) fed rats had hyperlipidaemia with increased TC, TG and LDL-C and decreased HDL-C. The TC, TG, LDL-C and HDL-C values were 149.9±8.8 mg/dl, 119.7±4.3 mg/dl, 105.7±5.0 mg/dl and 20.3±2.9 mg/dl respectively while the normal values are 70.6±3.1 mg/dl, 60.6±3.9 mg/dl, 18.3±1.2 mg/dl and 40.2±3.5 mg/dl for the same parameters. Intraperitoneal injection of CoQ_{10} for three weeks at a dose of 10 mg/kg daily had significantly alleviated the levels of TC, TG, LDL-C and HDL-C to 112.5±4.6 mg/dl, 87.3±6.1 mg/dl, 61.5±1.7 mg/dl and

33.5±1.7 mg/dl respectively [Modi *et al.* 2007]. CoQ_{10} has a key role in increasing fenofibrate activity in reducing the massive hypertriglyceridemia (MHTG) in patients. CoQ_{10} administered along with fenofibrate had shown to be efficacious in patients who were previously resistant to the treatment. CoQ_{10} supplementation alone has shown no improvements in the MHTG. CoQ_{10} when added 150 mg/day showed the lipid lowering activity in the group of patients who were on fenofibrate treatment. Even though the mechanism is unknown these results showed that CoQ_{10} is beneficial in lipid lowering [Cicero *et al.* 2005].

There were no known studies of the combination of EA and CoQ_{10} in atherogenic models or in patients with hyperlipidaemia. In our previous studies, the combination was shown to be effective in ameliorating the lipid peroxidation and elevated plasma lipids in STZ induced diabetic rats. EA and CoQ_{10} were reported to possess hypolipidemic action, moreover synergism existed between these agents in ameliorating triglyceride levels in STZ induced diabetic rats [Ratnam *et al.* 2008]. However, both these agents suffer from poor biopharmaceutical properties which affect the bioavailability remarkably. The poor biopharmaceutical properties of these antioxidants are limiting their clinical use [Ratnam *et al.* 2006]. The present work deals with the nanoparticulate approach for the combination of EA and CoQ_{10} in treating the hyperlipidaemia.

3.1.1. Aims

The objective of this part of the research program is to understand the effects of EA and CoQ_{10} combination in ameliorating hyperlipidaemia as well as the ability to protect the endothelium. The objectives will be achieved by the following specific aims:

- By developing appropriate formulations that can facilitate better peroral delivery with improved efficacy of poorly bioavailable EA and CoQ_{10} either individual or their combinations. In this case, we developed nano-co-encapsulated antioxidant particles of EA and CoQ_{10}.
- By evaluating the efficacy of the developed NanoCAPS in suitable animal model by measuring the pharmacological end points such as plasma lipid levels and endothelial functioning. In this case, we used high fat diet fed rat model.

3.2. Materials and Methods

All the experiments in this chapter were carried out at National Institute of Pharamaceutical Education and Research (NIPER), S.A.S. Nagar, India.

3.2.1. Nanoparticles

NanoCAPs of EA and CoQ_{10} were obtained by following the procedure presented in section 2.2.2.1 of Chapter 2.

3.2.2. Animals

Male Sprague–Dawley (SD) rats (180–200 g) were procured from the central animal facility of National Institute of Pharmaceutical Education and Research (NIPER), India. Animals were housed in standard polypropylene cages (three rats/cage) and maintained under controlled room temperature (22±2°C) and humidity (55±5%) with 12 h light and 12 h dark cycle. All the rats were fed with commercially available rat normal pellet diet (NPD) (Amrut Diet, New Delhi, India) and water *ad libitum*, prior to the dietary manipulation in treatment groups. The guidelines of committee for the purpose of control and supervision of experiments on animals (CPCSEA),

government of India were followed and prior permission was sought from the institutional animal ethics committee for conducting the study.

3.2.2. Development of experimental hyperlipidaemic model and treatment

Hyperlipidaemia was induced in rats by feeding high fat diet (HFD) for 4 weeks. The HFD comprises of 58% fat, 25% protein and 17% carbohydrate, as a percentage of total kcal. The exact composition with powdered NPD 365 g, lard 310 g, casein 250 g, cholesterol 10 g, vitamin and mineral mix 60 g, dl-methionine 3 g, yeast powder 1 g and sodium chloride 1 g was used to prepare 1 kg of HFD [Srinivasan *et al.* 2005].

Rats were divided into four groups (n=12 in each group). Except the control group which was fed NPD all the other group animals were fed HFD for the whole experimental period of 8 weeks. Groups receiving HFD were again divided into no treatment, and treatment groups. After 4 weeks, 2 groups of animals were treated either with EA and CoQ_{10} combination suspension (H+ECS) or NanoCAPs (H+NanoCAPs) at a dose of 25 mg/kg of both the antioxidants. The dosage forms were administered orally using an oral gavage needle and the treatment was carried out for 2 weeks till the end of 6^{th} week. Dosing frequency was once in three days in case of NanoCAPs and daily for the suspension group. Blood sampling was done after 4^{th}, 6^{th}, 7^{th} and 8^{th} week and various biochemical parameters related to hyperlipidaemia were studied. Six rats from each group were sacrificed at the end of the treatment, i.e., 6^{th} week and the endothelial relaxation was studied.

3.2.3. Estimation of biochemical parameters

Plasma was separated by centrifugation and was analysed for glucose (GOD-POD), triglycerides (GPO-POD) and total cholesterol (CHOD-POD)

levels using commercially available colorimetric diagnostic kits (Accurex Biomedical Pvt. Ltd., Thane, India).

3.2.4. Vascular tissue experiments

Six weeks from the start of the experiment, rats were sacrificed by cervical dislocation under mild ether anaesthesia; the thoracic aorta was quickly excised and placed in cold oxygenated (95% O_2-5% CO_2) Krebs'-Henseleit buffer (KHB). The composition of KHB was NaCl: 118, KCl: 4.7, $MgCl_2.2H_2O$: 1.2, $CaCl_2$: 2.6, KH_2PO_4: 1.2, $NaHCO_3$: 25 and glucose: 5.5 millimoles per liter. The thoracic aorta was cleaned off from adhering fat and adventitial tissue and 4-5 mm segments were cut. Care was taken not to stretch the tissue or damage the endothelium.

Aortic rings were suspended by a pair of stainless steel hooks in water-jacketed organ bath, filled with 10ml of oxygenated KHB maintained at 37°C. A resting tension of 2 g was applied to the aortic rings, which were then allowed to equilibrate for 2 h and the buffer was changed every 15 min. After the equilibration, tissues were pre-contracted with 10 μM phenylephrine (PE) and responses to increasing concentrations of acetyl choline (ACh) (1 nM to 10 μM) were recorded. All drugs were prepared according to their individual solubility and kept at -20 °C as 100 μl aliquots in concentrations of 10^{-2} and 10^{-3} M and they were diluted on the day of experiment. Stock solutions were prevented from repeated freezing and thawing to assure their stability. ACh mediated endothelium dependent vascular relaxations were expressed as percent of PE induced contraction [Arun *et al.* 2004].

3.2.5. Statistical analysis

Experimental values were expressed as mean ± standard error of mean (SEM). Statistical significance between groups was determined by

using one way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons.

3.3. Results

HFD fed rats had increased levels of plasma TC [Fig. 3.1] and LDL-C [Fig. 3.2] when compared with NPD fed rats after 4 weeks. HFD fed rats showed decreased levels of HDL-C [Fig. 3.3] and increased plasma triglycerides (TG) [Fig. 3.4] and plasma glucose levels [Fig. 3.5] than NPD rats after 4 weeks. These values were consistently different at 6th, 7th and 8th week. At 6th week in the end of the treatment H+ECS and H+NanoCAPs showed a reduction in the elevated plasma TC, LDL-C, TG and glucose levels but had no effect on the HDL-C levels [Fig. 3.1-3.5]. At 7th week the reduction in plasma TC was maintained by the suspension as well as NanoCAPs. On 8th week only H+NanoCAPs showed significant reduction in TC levels but not the H+ECS [Fig. 3.1]. In case of plasma LDL-C & HDL-C both the formulations showed similar effects [Fig. 3.2 & 3.3]. At 7th and 8th week there was no effect of ECS or NanoCAPs on the plasma TG levels [Fig. 3.4]. Plasma glucose levels were brought back to normal with the treatment of both the formulations and the effect was sustained until 8th week [Fig. 3.5]. The endothelium dependent relaxation was compromised in HFD fed rats and it was brought to normal levels with H+ECS as well as H+NanoCAPs [Fig. 3.6].

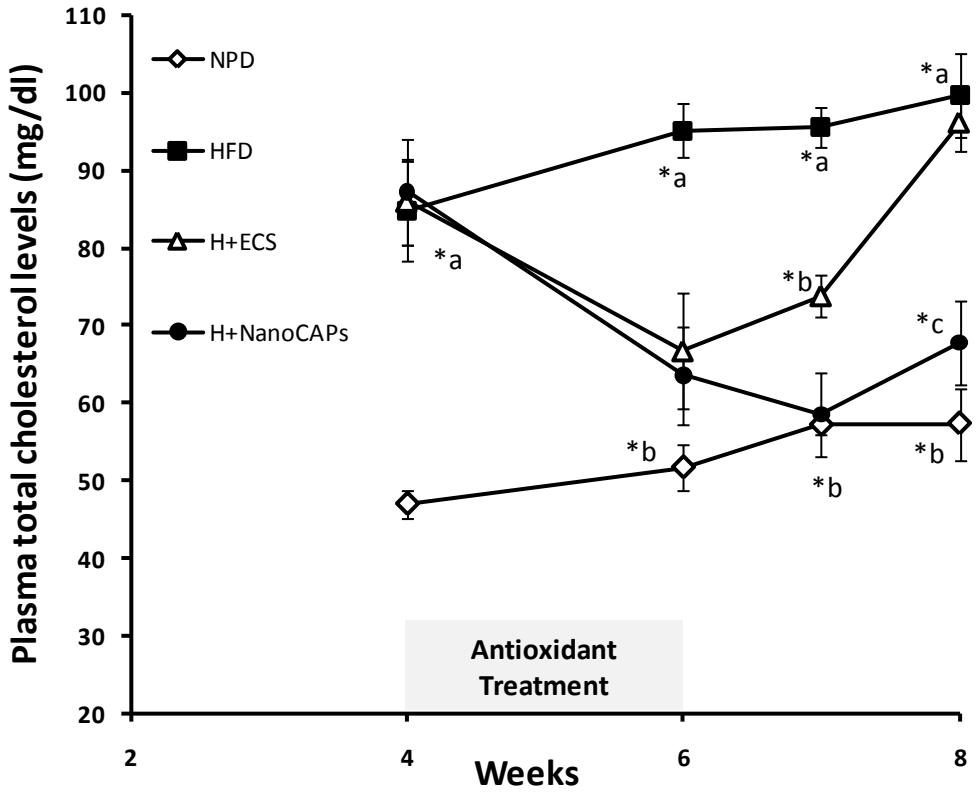


Fig. 3.1. The effect of suspension and NanoCAPs formulations of EA and CoQ_{10} combination on plasma total cholesterol levels. Treatment with the formulations was carried out between 4th and 6th week. Values are expressed as mean ± SEM (n=6); a versus NPD, b versus HFD, c versus H+ECS *p <0.001.

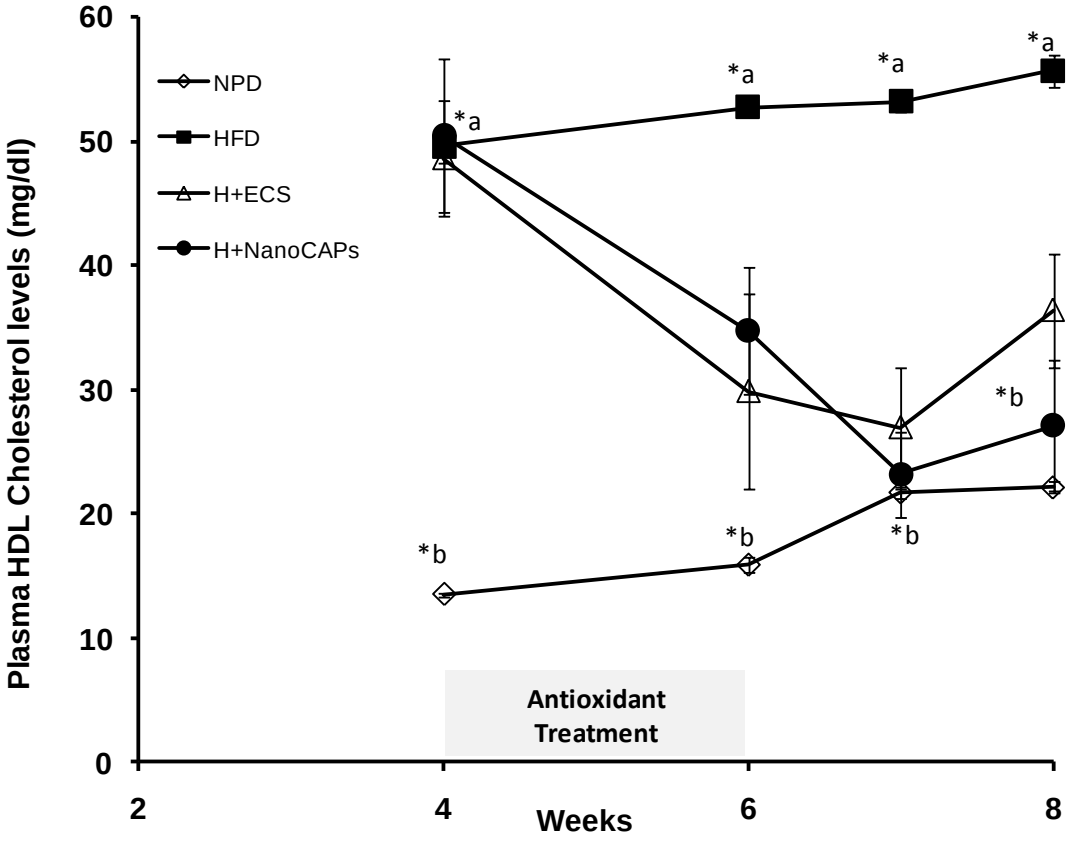


Fig. 3.2. The effect of suspension and NanoCAPs formulations of EA and CoQ_{10} combination on plasma LDL cholesterol levels. Treatment with the formulations was carried out between 4th and 6th week. Values are expressed as mean ± SEM (n=6); a versus NPD, b versus HFD, *p <0.001.

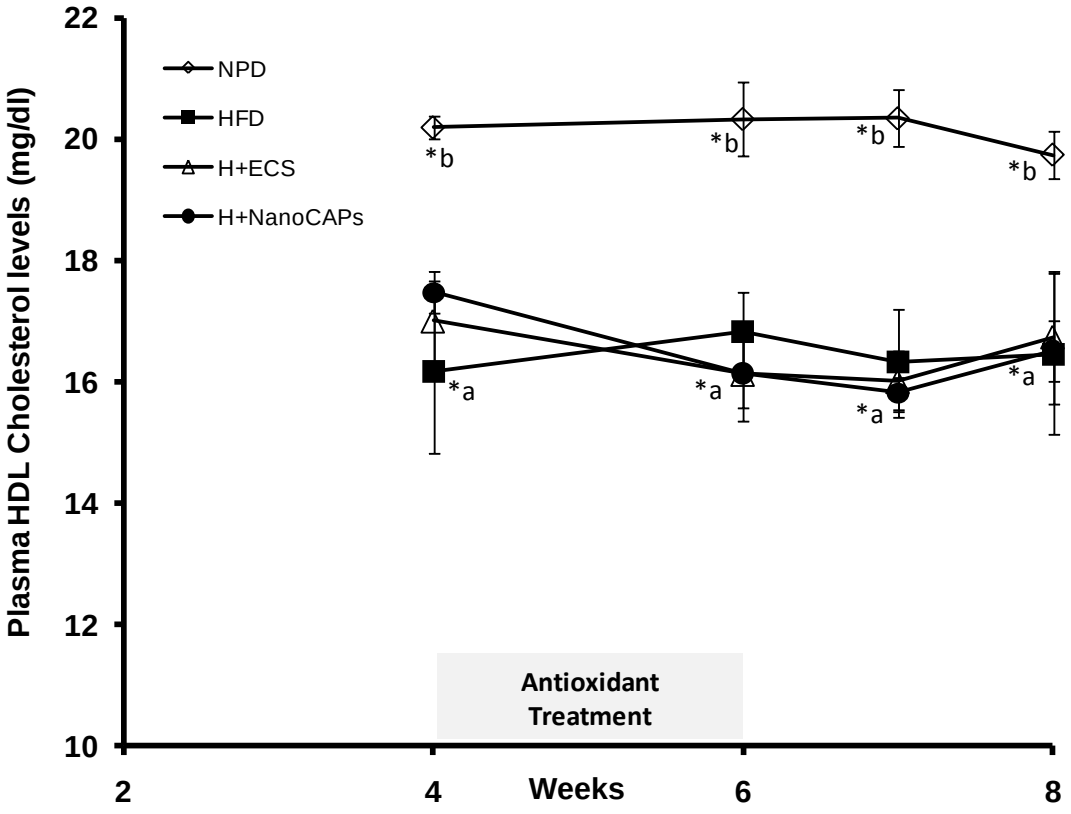


Fig. 3.3. The effect of suspension and NanoCAPs formulations of EA and CoQ_{10} combination on Plasma HDL-Cholesterol levels. Treatment with the formulations was carried out between 4th and 6th week. Values are expressed as mean ± SEM (n=6); a versus NPD, b versus HFD, *p <0.001.

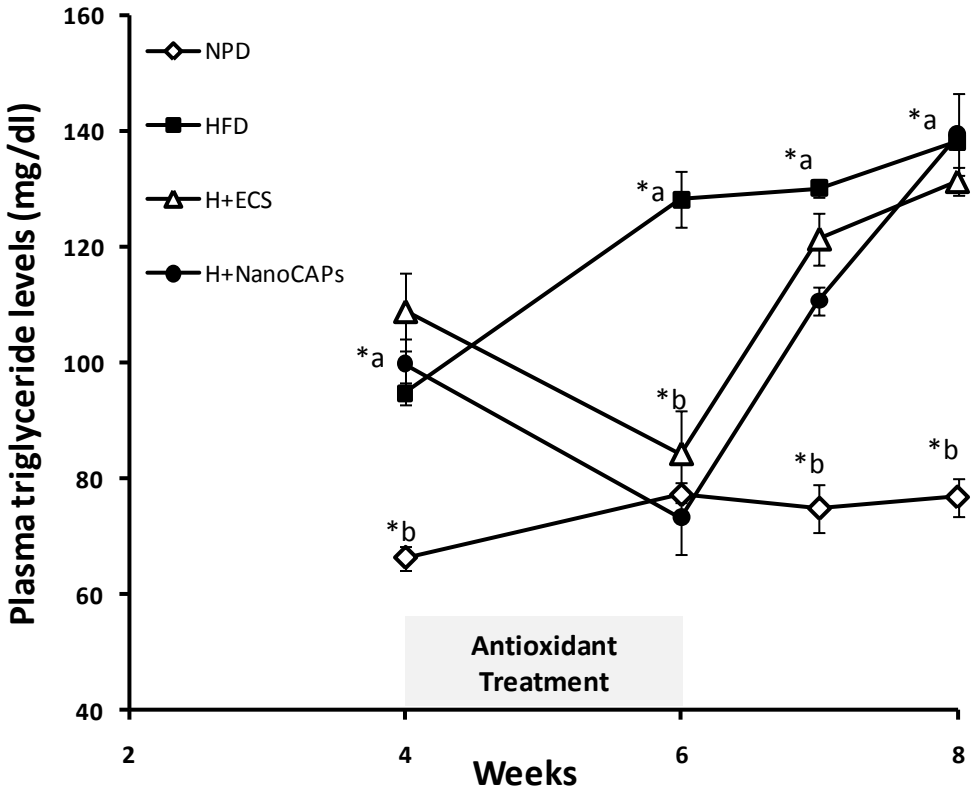


Fig. 3.4. The effect of suspension and NanoCAPs formulations of EA and CoQ_{10} combination on plasma triglyceride levels. The treatment with the formulations was carried out between 4th and 6th week. Values are expressed as mean ± SEM (n=6); a versus NPD, b versus HFD, *p <0.001.

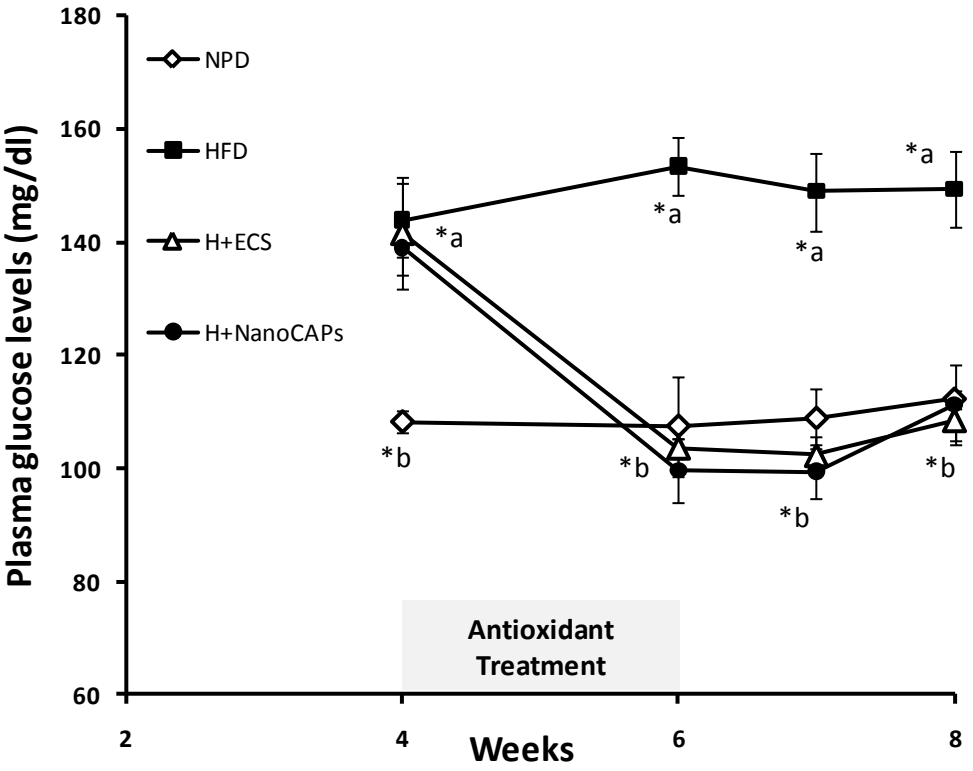


Fig. 3.5. The effect of suspension and NanoCAPs formulations of EA and CoQ_{10} combination on plasma glucose levels. Treatment with the formulations was carried out between 4th and 6th week. Values are expressed as mean ± SEM (n=6); a versus NPD, b versus HFD, *p <0.001.

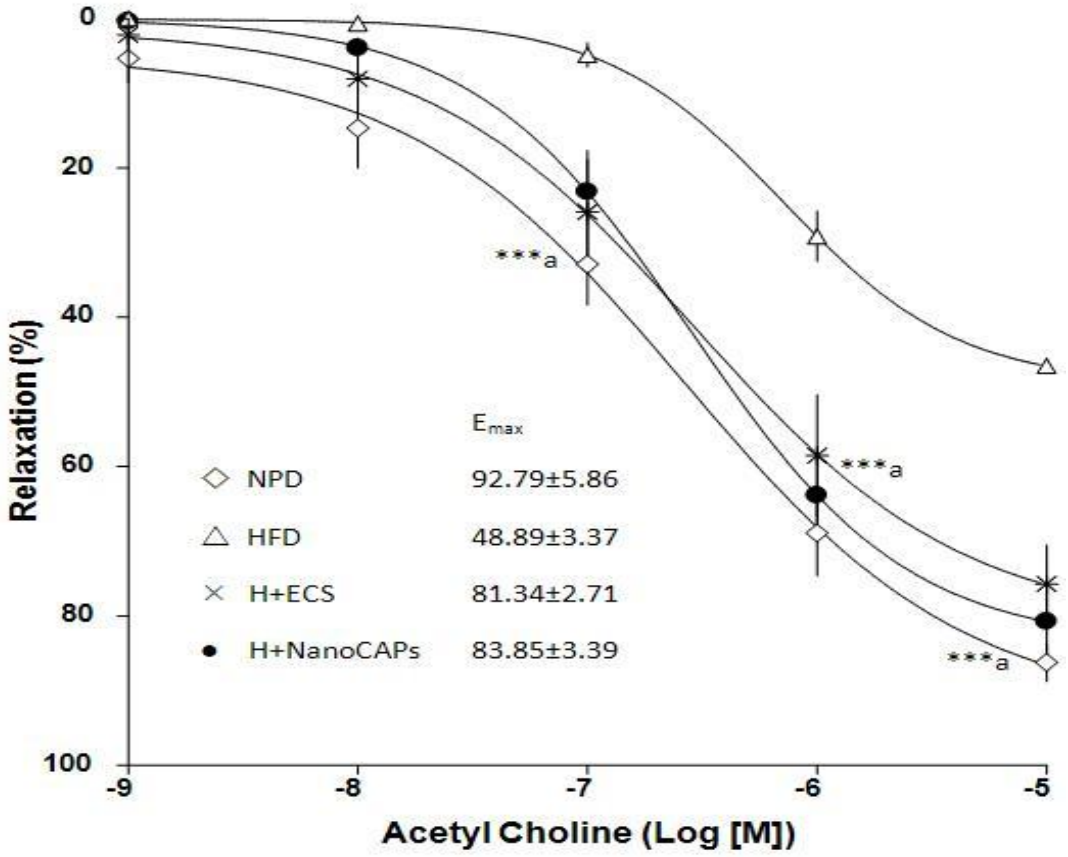


Fig. 3.6. Cumulative concentration response curves of rat aortic rings obtained from rats treated with suspension and NanoCAPs formulations of EA and CoQ_{10} combination. Values are expressed as mean ± SEM (n=5-6); a versus HFD, ***p <0.001.

3.4. Discussion

In the present study, after 4 weeks of HFD feeding, rats were found with elevated levels of plasma TC, LDL-C, TG and glucose [Fig.3.1, 3.2, 3.4, & 3.5] but with decreased levels of HDL-C [Fig. 3.3]. Hypercholesterolaemia may be attributed to increased dietary cholesterol absorption from the small intestine following the intake of cholesterol rich HFD [Xie *et al.* 2009]. The hypertriglyceridaemia observed in HFD fed rats may be due to increased absorption and formation of TG in the enterocytes as chylomicrons following exogenous consumption of diet rich in fat or through increased endogenous production of TG-enriched hepatic VLDL and decreased TG uptake in peripheral tissues. As a result of compensatory hyperinsulinaemia, mild hyperglycaemia persists in the HFD fed rats [Srinivasan *et al.* 2004].

The EA in the combination of agents used might be responsible in preventing the absorption of the cholesterol from the gut [Lei *et al.* 2007]. Reports on CoQ_{10} supplementation had shown no effect on cholesterol lowering [Sohet *et al.* 2009; Witting *et al.* 2000]. The ECS and NanoCAPs showed same level of reduction in plasma TC levels at 6th week. The levels were kept below the HFD controls even after cessation of treatment with suspension for one week (till 7th week) and with nanoparticles for 2 weeks (till 8th week) [Fig. 3.1]. During the administration (4th to 6th week) of ECS and the NanoCAPs, the absorption of the cholesterol might have been restricted. The inhibition of cholesterol absorption produces a state of relative cholesterol deficiency followed by cholesterol biosynthesis and up regulation of LDL receptors [Brufau *et al.* 2008]. Cholesterol is mainly transferred to the body via LDL particles. LDL particles are formed from the VLDL particles, after losing the triglyceride content and becoming rich in cholesterol. Due to the up regulation of the LDL receptors it might be possible that the LDL-C

levels in blood remained low even after the cessation of the treatment (7th & 8th weeks) [Fig. 3.2]. As the LDLs are the main transporters of the cholesterol the TC levels were also affected by the changes in LDL-C levels. The difference in the levels of LDL-C and TC levels can be attributed to the VLDL-C where in the increased cholesterol biosynthesis have increased their levels after stoppage of the treatment. This difference was not clearly seen when the formulations were administered (at 6th week), but when the administration was stopped (after 6th week) the TC levels reached the HFD control values but LDL-C levels were comparable to the control values even at 8th week. HDL-C levels were not influenced by ECS or NanoCAPs treatment [Fig. 3.3].

Nanoparticles have showed comparable reductions to the suspension, in the TC levels although the nanoparticles were administered at 3 times lower frequency and dose than the suspension. This suggests that the prolonged effect of nanoparticles on sustaining the elevated cholesterol levels could be due to the sustained release of EA and CoQ_{10} for prolonged durations from the nanoparticles [Fig. 3.1 &3.2]. This sustained action of nanoparticles is in accordance with the previous reports with nanoparticles where nanoparticles sustained the release of the loaded therapeutic ingredient to result in improved delivery and better therapeutic end point [Sonaje *et al.* 2007].

Plasma TG levels were reduced efficiently with both forms of the treatment at 6th week [Fig. 3.4.]. Triglycerides are the major components of VLDL and chylomicrons and play an important role in metabolism as energy sources and transporters of dietary fat. In the intestine, triglycerides are split into monoacylglycerol and free fatty acids by lipolysis, with the secretion of lipases and bile, which are subsequently moved to absorptive enterocytes

lining the intestine. The triglycerides are rebuilt in the enterocytes from their fragments together with cholesterol and proteins to form chylomicrons. These are excreted from the cells and collected by the lacteals and later on mixed with blood. Literature suggests that some natural antioxidants possess inhibitory properties on fat absorption [Lampe 1999]. Pomegranate leaf extract, which is rich in ellagic acid, has been shown to reduce the plasma TG in HFD fed obese mice. Authors suggest that the effects appear to be partly mediated by inhibiting the pancreatic lipase activity and suppressing energy intake [Lei *et al.* 2007]. We have previously demonstrated the ability of the combination of EA and CoQ_{10} in ameliorating the triglyceride levels in STZ induced diabetic rats [Ratnam *et al.* 2008]. Together these two agents efficiently reduced the triglyceride levels when delivered as suspension as well as nanoparticles with reduced frequency and dose. However, there was no sustained action seen after the cessation of treatment as in cholesterol levels. The possible reason can be related to the breakdown, absorption and resynthesis of triglycerides in the enterocytes. When there was co-administration of EA & CoQ_{10} with HFD feeding these antioxidants might have interfered in one of the above said three processes in the enterocytes. When the treatment with EA & CoQ_{10} was ceased there might not be sufficient levels available to interfere with the absorption of the TG at the enterocytes.

In HFD fed rats, the glucose levels are high in comparison to the normal rats suggesting a state of insulin resistance. This is considered to be due to the obesity but not due to the recently absorbed fat from the diet, suggesting a feedback mechanism which might have developed with time. High serum lipid levels may inhibit the glycolysis and reduce the insulin effect causing the insulin resistance. Excessive free fatty acids and other

metabolic products of the fats in the liver reduce the insulin sensitivity of the hepatocytes and distort the glucose metabolism [Akiyama *et al.* 1996]. Plasma glucose levels were reduced with H+ECS and H+NanoCAPs. The effect was sustained even after the treatment was stopped at least for 2 more weeks [Fig 3.5]. Both the groups showed same levels of reducing effects even though their dosing frequencies were different. When the plasma lipid levels were brought down with the treatment of EA & CoQ_{10} the plasma glucose levels were also brought down. During the 2 weeks of sustaining the lipid levels in the circulation the glucose metabolism might have brought back to normal. Till 8th week except the TG levels, LDL-C levels were comparable to the NPD animals, which might be the reason for the sustained levels of glucose even at the 8th week.

In hyperlipidaemia the antioxidant activity is reduced and the LDL particles become oxidised, which pose a great risk for the endothelium. There is substantial evidence that many endothelial functions are sensitive to the presence of ROS and subsequent oxidative stress. Endothelium is responsible for the production of eNOS which is responsible for NO synthesis ultimately showing a relaxing effect on the vascular smooth muscle. The activity of eNOS is inhibited by the circulating amino acid asymmetrical dimethylarginine, the levels of which are increased in hypercholesterolemia [Vogel *et al.* 1998]. Pomegranate juice in which EA is one of the major constituent has been shown to have effects on eNOS and oxidation sensitive genes [Nigris *et al.* 2005]. CoQ_{10} is known to have protective effects on coronary endothelial function from ischemia reperfusion injury [Yokoyama *et al.* 1996]. Both these agents act as antioxidants and can help improving the endothelial dysfunction caused by HFD. The suspension as well as nanoparticle formulations were equally effective in protecting the

endothelium, which was evident from the over 80% relaxation when compared to the HFD rats where the relaxation is only 49±3% irrespective of their frequency of administration [Fig. 3.6]. The nanoparticles are reported to be engulfed by the endothelial cells [Davda and Labhasetwar 2002], thus more efficient action of the EA and CoQ_{10} with the nanoparticles was seen at 66% reduced doses in comparison to the suspension group.

3.5. Conclusion

In conclusion, EA and CoQ_{10} combination can be used to prevent the hypercholesterolaemia, hypertriglyceridaemia and hyperglycaemia although the mechanisms of action need to be studied elaborately. The combination is also protective to the endothelium, which can be very beneficial in prevention of atherosclerosis. The nanoparticulate approach was proven to be successful and efficacious when compared to the simple suspension form of the same combination at much lower doses. These NanoCAPs can be of great use for the treatment of hyperlipidaemia in metabolic syndrome or diabetic dyslipidaemia.

4. EVALUATION OF COENZYME Q10 NANOPARTICLES AND CURCUMIN NANOPARTICLES IN DIABETIC COMPLICATIONS DEVELOPED IN THE RAT

4.1. Introduction

Vascular inflammation and cardiovascular disease (CVD) are the leading causes of morbidity and mortality in the diabetic population and remain major public-health issues [Frankel *et al.* 2011]. Factors such as proinflammatory cytokines, glycosylation of proteins, and oxidative stress, are thought to contribute to the development of vascular inflammation in diabetes. Inflammatory mediators like tumour necrosis factor α (TNF-α), interleukin-6 (IL-6) were known to be elevated in diabetes induced animals and patients in T1D and T2D. Complications develop in T1D as a result of increased oxidative stress as well as inflammation. A study used Chinese Guizhou minipigs for the development of diabetic complications by injecting streptozotocin (STZ). After six months the measurement of the levels of TNF-α and IL-6 along with other inflammatory markers showed increased levels of the proinflammatory cytokines in the aortic intima [Lu *et al.* 2007]. TNF-α is a proinflammatory cytokine that is clearly implicated in the pathogenesis of autoimmune diseases and other inflammatory disorders. Evidence suggests that neutralization of TNF-α can improve insulin sensitivity [Hotamisligil 1999]. Importantly, results from knockout mice deficient in TNF-α or its receptors have suggested that TNF-α has a role in regulating *in vivo* insulin sensitivity. However, nullifying TNF-α action might only partially protect against obesity-induced insulin resistance in mice. Multiple mechanisms have been suggested to account for the metabolic effects of TNF-α. These include the down regulation of genes that are required for normal insulin action, direct effects on insulin signalling, induction of elevated free fatty acids via stimulation of lipolysis, and negative regulation of PPARγ, an

important insulin-sensitizing nuclear receptor. Although current evidence suggests that neutralizing TNF-α in T2D subjects is not sufficient to cause metabolic improvement, it is still probable that TNF-α is a contributing factor in common metabolic disturbances such as insulin resistance and dyslipidaemia which are seen in T1D [Moller 2000]. The role of IL-6 in vascular inflammation has been shown in studies with IL-6 knockout mice which exhibit resistance to splanchnic artery occlusion shock. Anti–IL-6 therapy significantly prevents the inflammatory process in mice, and other studies have shown increased levels of lipid peroxidation and inflammation in mice that overexpress IL-6 [Castelnau *et al.* 1998]. CRP is another marker of inflammation, blood levels of which rise dramatically in inflammatory conditions [Colhoun *et al.* 2002]. Higher levels of CRP predict cardiovascular disease in asymptomatic men and in women in the general population, indicating a possible role for inflammation in the etiology of cardiovascular disease [Koenig *et al.* 1999].

The relationship between diabetes and hyperlipidaemia is a well recognised phenomenon [Valensi and Picard 2011]. Hypercholesterolemia is a common feature observed in diabetes contributing to the high prevalence of coronary heart disease. Many compositional abnormalities of the lipoproteins VLDL, LDL and HDL have been found in diabetic patients. Lipid levels are altered in both types of diabetes mellitus. In T1D typical LDL-C and HDL-C levels are comparable to normal levels but the triglyceride levels are raised because of the poor glycaemic control. Poor glycaemic control leads to a reduction in the activity of LPL followed by the inability to clear chylomicrons and VLDL results in the increased levels of triglycerides. When the glycaemic control is very poor HDL cholesterol levels

may also be decreased and LDL cholesterol levels may be elevated [O'Brien *et al.* 1998].

Observations suggest that the levels of CoQ_{10} were decreased in STZ induced diabetic conditions [Kucharska *et al.* 2000]. Beneficial effects of CoQ_{10} in humans occur through various mechanisms including the possibility of immunomodulation other than its antioxidative activity. TNF-α secretion was significantly decreased with CoQ_{10} administration in human peripheral blood mononuclear cells [Bessler *et al.* 2009]. Another study found a remarkable effect of vitamin E and CoQ_{10} co-supplementation on markers of vascular inflammation as indicated by CRP. Dietary vitamin E at a dose as low as 250 IU/kg diet to baboons had achieved a reduction by nearly 50% in serum CRP concentrations. Addition of 2g CoQ_{10}/kg diet to the vitamin E supplementation resulted in further 20% decrease in CRP levels [Wang *et al.* 2004]. CoQ_{10} is beneficial in the management of diabetic dyslipideamia. It has shown to be beneficial alone and is useful as an alternative to statins in patients intolerant to the statin therapy [Caso *et al.* 2007].

Diabetogenesis occurs through slow damage to the islet cells with time involving proinflammatory cytokines. Cytokines induce oxidative stress to result in the destruction of islet cells. Curcumin was shown in a study to be able to prevent cytokine induced cell death *in vitro* and to prevent diabetogenesis in STZ induced diabetes in mice. Although these researchers showed the benefits of curcumin they stated that it was not feasible for the oral delivery of curcumin and their studies were carried out intraperitonially at the dose of 7.5 mg/kg [Kanitkar *et al.* 2008]. Curcumin is a potent antioxidant and an anti-inflammatory agent. There are many studies suggesting the role of curcumin in diabetes, where it has shown to be effective against delaying the development of cataract and nephropathy in

STZ induced diabetes [Jain *et al.* 2009]. Curcumin was shown to be very beneficial in diabetic retinopathy in STZ induced diabetic Wistar albino rats. The study showed beneficial effects of curcumin through hypoglycaemic, antioxidative and anti-inflammatory mechanisms. TNF-α levels in the retina of curcumin treated diabetic rats were 2.5 times lower than the diabetic control [Gupta *et al.* 2011]. Similar studies including pancreatic islet cell regeneration along with antioxidative and anti-inflammatory actions revealed that curcumin is a potential therapy for diabetes and its complications [El-Azab *et al.* 2011].

Research nowadays is focused on the manufacture of different kinds of formulations of curcumin to overcome its poor biopharmaceutical properties and testing them in different animal and cell culture models [Bisht *et al.* 2007, Belcaro *et al.* 2010, Munjal *et al.* 2011]. A group studied the role of curcumin loaded poly(ε-caprolactone) nanofibres in diabetic wound dressing. The fibres showed slow release of curcumin for up to 72h and shortened the duration of diabetic wound healing with antioxidant and anti-inflammatory activities. These nanofibres reduced inflammatory induction which was evidenced by low levels of IL-6 release from mouse monocyte-macrophages seeded on the fibres following stimulation by lipopolysaccharide [Merrel *et al.* 2009]. Similarly another delivery approach showed the significant increase in bioavailability of the curcumin loaded PLGA nanoparticles over suspension form. Curcumin loaded nanoparticles showed at least 9 times increased bioavailability and sustained release for about 48h [Shaikh *et al.* 2009].

4.1.1. Aim

The objective of this part of the research program is to understand the role of CoQ_{10} and curcumin in alleviating the proinflammatory markers in

diabetic complications. The objectives will be achieved by the following specific aims:

- By developing appropriate formulations that can facilitate better peroral delivery with improved efficacy of poorly bioavailable CoQ_{10} and curcumin. In this case, we developed CoQ_{10} and curcumin nanoparticles.
- By evaluating the efficacy of the developed CoQ_{10} and curcumin nanoparticles in suitable animal model by measuring the pharmacological end points such as proinflammatory markers and lipid levels. In this case we have used STZ induced diabetic rats.

4.2. Materials and methods

CoQ_{10} nanoparticles, curcumin nanoparticles and blank nanoparticles stabilised with PVA and DMAB were obtained following the procedures described in section 2.2.2.2 of chapter 2.

Diabetic complication studies were carried out in male Sprague Dawley rats weighing between 250-300g with n=6 animals per group. All procedures on animals were performed according to the United Kingdom Home Office Guide on the Operation of Animals (Scientific Procedures) Act 1986. All rats were allowed to stabilize for 15 days and housed at 12 h dark-light cycle with access to food and water *ad libitum*. Animals were divided into 6 groups; group I comprised of normal animals (NC), groups II-VI animals were made diabetic by a single intraperitoneal injection of STZ (in 10 mM ice cold citrate buffer adjusted to pH 4.5) at a dose of 55 mg/kg. From the 16^{th} day of diabetes induction group II (STZ) diabetic animals received vehicle (water), group III (STZ+CoN) received 100 mg/kg/day CoQ_{10} nanoparticles, group IV (STZ+CuN) received 100 mg/kg/day curcumin nanoparticles, group V (STZ+BD) received blank PLGA nanoparticles

stabilised with DMAB and group VI (STZ+BP) received blank PLGA nanoparticles stabilised with PVA. All the formulations were given orally using an oral gavage needle. After 31 days from the diabetes induction, animals were sacrificed and blood was collected. Blood samples were centrifuged at 10000 rpm for 5 min and plasma was separated and stored at -20°C until further analysis. Evaluation of treatment efficacy was carried out by assaying the serum levels of pro-inflammatory and lipid markers in the blood samples which comprised of TNF-α, IL-6, CRP, cholesterol, TG and HDL, the levels of which were estimated using their respective assay kits. The results were expressed as graphs and the statistical analysis was carried out using one way analysis of variance followed by comparison with the positive control group to other groups using Holm-Sidak method using sigma stat software.

4.3. Results

STZ group diabetic rats were found with elevated levels of plasma TNF-α [Fig. 4.2], TG [Fig. 4.4] and TC [Fig.4.5] significantly in comparison to the NC rats after one month. HDL-C levels were significantly decreased in the STZ group in comparison to NC group [Fig. 4.6]. IL-6 and CRP levels in STZ group are not significantly different from the NC group [Fig 4.1 & 4.3].

Curcumin nanoparticles significantly lowered IL-6, TNF-α and CRP levels in diabetic rats in comparison to the STZ diabetic rats at the end of the study [Fig. 4.1&4.2]. CRP levels were reduced significantly after 2 weeks of treatment with CoQ_{10} nanoparticles treated diabetic rats [Fig. 4.3]. Plasma TG levels were raised significantly in diabetic rats and brought back to normal levels with CoQ_{10} as well as curcumin nanoparticles treated diabetic rats [Fig. 4.4]. Plasma TC levels were increased remarkably in the diabetic control group; CoQ_{10} as well as curcumin nanoparticles are efficient in attenuating

the cholesterol levels to normal levels [Fig. 4.5]. Plasma HDL-C levels were reduced in diabetic rats, which were raised significantly in the CoQ_{10} as well as curcumin nanoparticles treated rats [Fig. 4.6]. Blank nanoparticles treated, either DMAB stabilised (STZ+BD group) or PVA stabilised (STZ+BP group), diabetic rats showed similar levels as the diabetic control showing no effect of the blank nanoparticles on all the measured parameters. We have not used plain curcumin and CoQ_{10} suspension controls owing to their poor deliverability because of poor solubility, and poor oral bioavailability [Shaikh *et al.* 2009, Ankola *et al.* 2007].

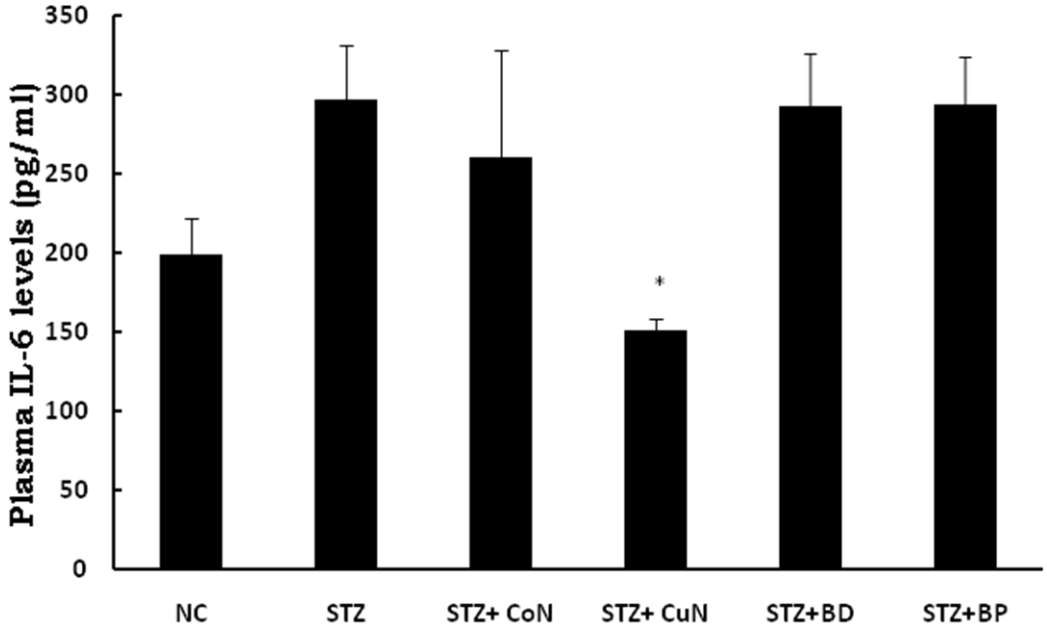


Figure 4.1. Plasma interleukin 6 levels. NC – non diabetic negative control, STZ – diabetic positive control developed by injecting STZ, STZ+CoN – CoQ_{10} nanoparticles treated diabetic rats, STZ+CuN–curcumin nanoparticles treated diabetic rats, STZ+BD – DMAB stabilised blank nanoparticles treated diabetic rats, STZ+BP – PVA stabilised blank nanoparticles treated diabetic rats. Values are depicted as mean ± S.E.M. * $p < 0.05$ vs STZ group.

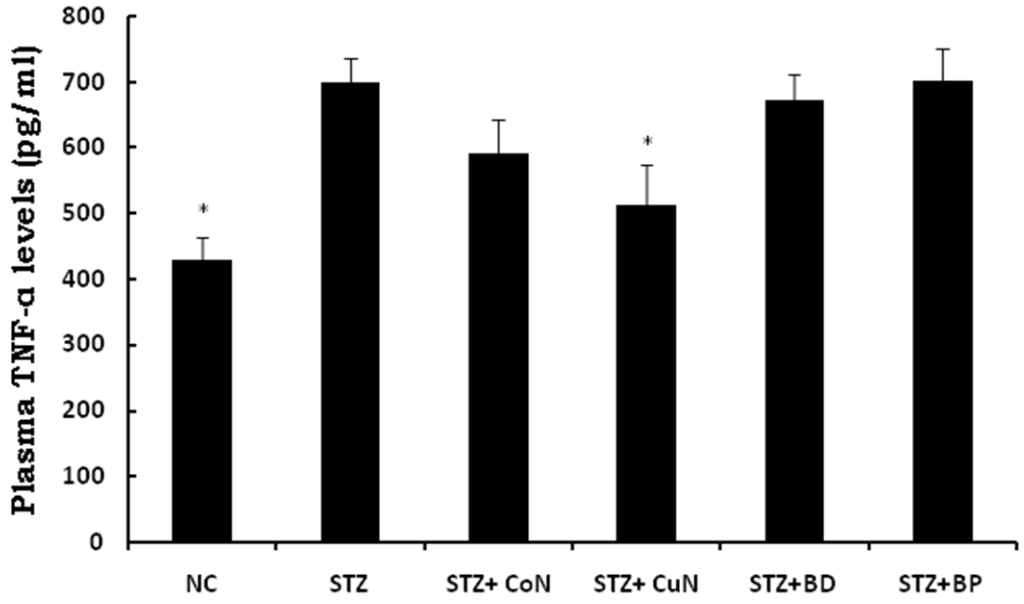


Figure 4.2. Plasma TNF-α levels. NC – non diabetic negative control, STZ – diabetic positive control developed by injecting STZ, STZ+CoN–CoQ_{10} nanoparticles treated diabetic rats, STZ+CuN – curcumin nanoparticles treated diabetic rats, STZ+BD – DMAB stabilised blank nanoparticles treated diabetic rats, STZ+BP – PVA stabilised blank nanoparticles treated diabetic rats. Values are depicted as mean ± S.E.M. * p <0.05 vs STZ group.

Figure 4.3. Plasma C-reactive protein levels. NC – non diabetic negative control, STZ – diabetic positive control developed by injecting STZ, STZ+CoN – CoQ_{10} nanoparticles treated diabetic rats, STZ+CuN – curcumin nanoparticles treated diabetic rats, STZ+BD – DMAB stabilised blank nanoparticles treated diabetic rats, STZ+BP – PVA stabilised blank nanoparticles treated diabetic rats. Values are depicted as mean ± S.E.M. * $p < 0.05$ vs STZ group.

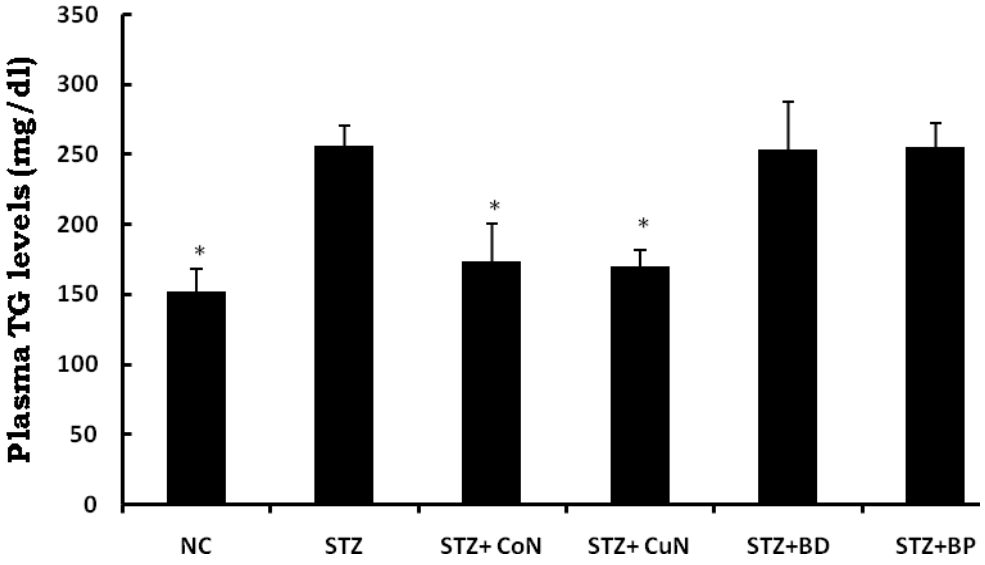


Figure 4.4. Plasma triglyceride levels. NC – non diabetic negative control, STZ – diabetic positive control developed by injecting STZ, STZ+CoN – CoQ_{10} nanoparticles treated diabetic rats, STZ+CuN – curcumin nanoparticles treated diabetic rats, STZ+BD – DMAB stabilised blank nanoparticles treated diabetic rats, STZ+BP – PVA stabilised blank nanoparticles treated diabetic rats. Values are depicted as mean ± S.E.M. * $p < 0.05$ vs STZ group.

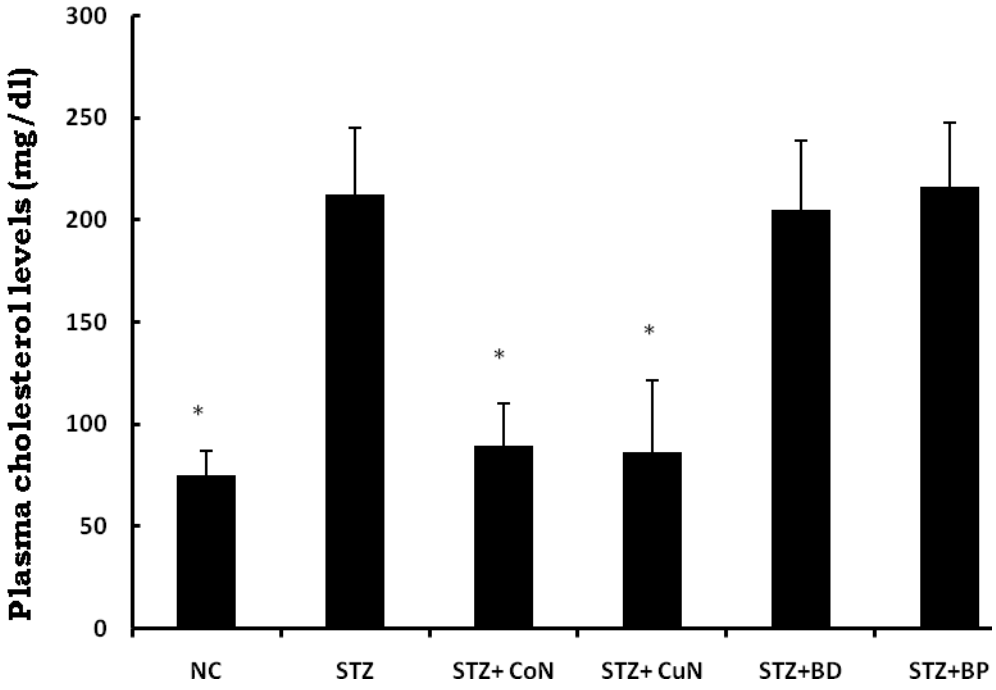


Figure 4.5. Plasma cholesterol levels. NC – non diabetic negative control, STZ – diabetic positive control developed by injecting STZ, STZ+CoN – CoQ_{10} nanoparticles treated diabetic rats, STZ+CuN – curcumin nanoparticles treated diabetic rats, STZ+BD – DMAB stabilised blank nanoparticles treated diabetic rats, STZ+BP – PVA stabilised blank nanoparticles treated diabetic rats. Values are depicted as mean ± S.E.M. * $p < 0.05$ vs STZ group.

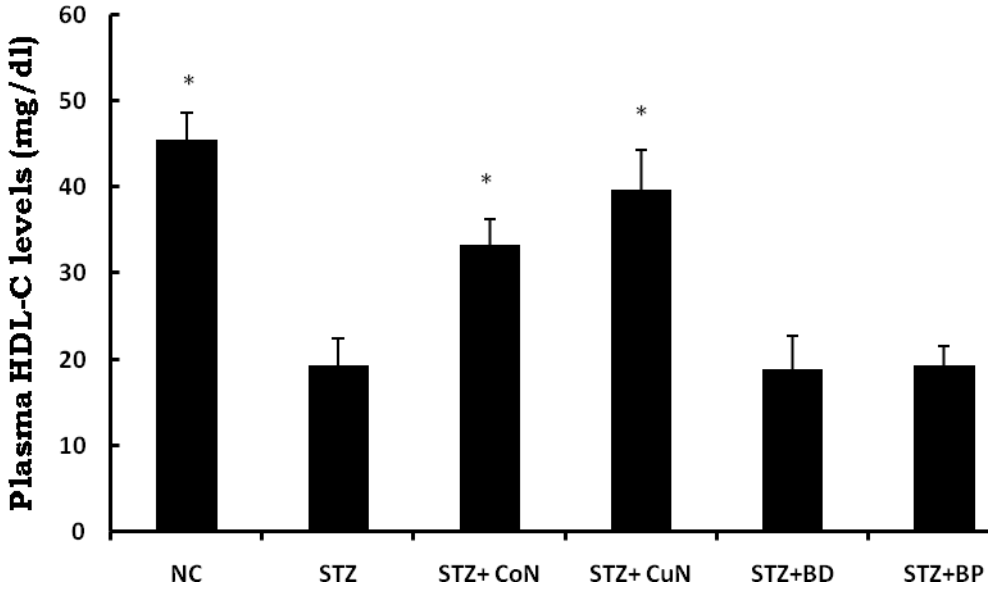


Figure 4.6. Plasma HDL-C levels. NC – non diabetic negative control, STZ – diabetic positive control developed by injecting STZ, STZ+CoN – CoQ_{10} nanoparticles treated diabetic rats, STZ+CuN – curcumin nanoparticles treated diabetic rats, STZ+BD – DMAB stabilised blank nanoparticles treated diabetic rats, STZ+BP – PVA stabilised blank nanoparticles treated diabetic rats. Values are depicted as mean ± S.E.M. * p <0.05 vs STZ group.

4.4. Discussion

It is well established that inflammation is the major cause in the development and progression of vascular diseases. In our study proinflammatory cytokine TNF-α levels in plasma increased significantly with STZ injection compared to the normal rats. This is in accordance with the literature reports suggesting increases in inflammatory cytokine levels in experimental diabetes incuded by STZ in Chinese Guizhou miniature swine [Lu *et al.* 2007] and rodents [Jain *et al.* 2009]. IL-6 and CRP levels rose slightly but were not significantly different to the normal rats. This could be due to short duration of the study i.e. 4 weeks, however with loger durations (7 or more weeks) after diabetes induction IL-6 and CRP levels also increase in experimental diabetes [Lu *et al.* 2007, Jain *et al.* 2009].

In the present study, TNF-α levels were slightly reduced with CoQ_{10} nanoparticles treatment in diabetic rats however they were not significant and no change in the IL-6 levels was seen in comparison to the diabetic control group [Fig. 4.1 & 4.2]. This observation is supported by the literature report where TNF-α and IL-6 levels were unaltered with 100 mg/day supplementation of CoQ_{10} in humans [Gokbel *et al.* 2010]. Figure 4.3 shows decreased levels of CRP with both the treatments suggesting the beneficiary role of CoQ_{10} loaded nanoparticles in the diabetes. There were no reports in the literature about the effects of CoQ_{10} on CRP levels in diabetes.

Nanoparticles of curcumin were very effective in alleviating the raised TNF-α, IL-6 and CRP levels in the STZ induced diabetic rats [Fig. 4.1-4.3]. Our results are in agreement with the literature reports where curcumin has shown its strong anti-inflammatory effects in rats and cell culture model. Curcumin was used with olive oil to serve the purpose of the vehicle to overcome the deliverability problem associated with curcumin in these

studies [Jain *et al.* 2009]. The anti-inflammatory effects of curcumin can be mediated through oxidative stress dependent or independent pathways [Kowluru *et al.* 2007, Sandur *et al.* 2007].

In the present study, CoQ_{10} loaded as well as curcumin loaded nanoparticles showed statistically significant reductions in plasma TG levels, TC levels and increased the HDL-C levels in diabetic rats [Fig 4.4-4.6]. CoQ_{10} nanoparticles have shown reductions in plasma TG and TC in our previous study using STZ induced diabetic rats at even much lower dose 50 mg/kg [Ratnam *et al.* 2008]. In humans 150 mg/day CoQ_{10} in combination with fenofibrate modulated the effect of fenofibrate in lowering the massive hypertrigyceridaemia [Cicero *et al.* 2005]. The lipid lowering effects of CoQ_{10} can be attributed to its direct effect on mitochondria to increase fatty acid oxidation, an antioxidant effect that could decrease oxidative stress, and/or a direct vascular effect that might have led to increased lipolysis of triglyceride rich lipoproteins [Modi *et al.* 2007, Cicero *et al.* 2005]. Literature supports that curcumin was effective against hyperlipidaemia developed in the streptozotocin induced diabetic rats [Babu & Srinivasan 1997] and high fat diet fed hamsters [Jang *et al.* 2008]. The hypolipidaemic action of the dietary curcumin is believed to be mediated by the increase in activity of hepatic cholesterol-7a-hydroxylase suggesting a higher rate of cholesterol catabolism [Babu & Srinivasan 1997].

The present study illustrates the use of novel delivery strategy using nanoparticles for the enhancement of deliverability of CoQ_{10} and curcumin. CoQ_{10} and curcumin faced deliverability problems due to poor solubility while attempting to deliver through oral route [Modi *et al.* 2007, Jain *et al.* 2009]. Some researchers have avoided the oral route and studied the effects of these antioxidants through other routes [Modi *et al.* 2007, Kanitkar *et al.*

2008], not many researchers instead of avoiding oral route applied different delivery strategies to make the oral delivery of these agents possible. The simplest approach is to use oil based formulations of CoQ_{10} and curcumin [Zmitek *et al.* 2008a, Jain *et al.* 2009] which result in only slight increases in bioavailability [Bhagavan and Chopra 2007], however, better efficacy than the suspension formulations. The nanoparticle approach is a newer and better approach for the improvement of deliverability of CoQ_{10} and curcumin. Nanoparticle approach offered enhanced uptake of CoQ_{10} in comparison to the oil based formulation during the in situ uptake studies performed using rat intestine [Ankola *et al.* 2007]. Curcumin nanoparticles showed multi-fold increase in bioavailability than the curcumin+absorption enhancer piperine formulation in a study [Shaikh *et al.* 2009]. Nanoparticles show better bioavailability and therapeutic efficacy in comparison to other approaches for CoQ_{10} and curcumin.

Plasma TC, LDL-C and TG levels were reduced with the NanoCAPs of EA and CoQ_{10} [Fig. 3.1, 3.2 & 3.4, chapter 3] in hyperlipidaemic rats and with the treatment of CoQ_{10} or curcumin nanoparticles in STZ diabetic rats. Although the two experiments represent the development of hyperlipideamia in two different ways the NanoCAPs, CoQ_{10} and curcumin nanoparticles used in our studies showed reduction in the plasma TC, LDL-C and TG levels. In high fat diet fed rats the increase in the plasma lipid levels is because of the fat enriched food. STZ induced diabetic rats represent the higher lipid levels because of the poor glycaemic control. It might be possible that the NanoCAPs inhibit the absorption of the fats in HFD fed rats, but the mechanism of lipid lowereing in STZ induced diabetes needs to be understood. NanoCAPs had no effect on HDL-C levels in HFD fed rats [Fig 3.3] but CoQ_{10} and curcumin nanoparticles showed increased HDL-C levels

in the diabetic rats [Fig 4.6]. More studies are needed to understand the mechanism of these antioxidants on the lipid levels.

Blank nanoparticles are used as controls in the present study are DMAB stabilised and PVA stabilised nanoparticles. DMAB stabilised blank nanoparticles served the purpose of negative controls for the CoQ_{10} loaded nanoparticles and PVA stabilised blank nanoparticles served the purpose of negative controls for the curcumin loaded nanoparticles. These blank nanoparticles showed similar levels of all the parameters studied suggesting that the polymer PLGA and stabilisers DMAB and PVA has no significant effect.

4.5. Conclusion

CoQ_{10} nanoparticles are beneficial in reducing the elevated levels of TG, TC and increasing the decreased levels of HDL-C in diabetic rats. Curcumin nanoparticles showed significant normalisation of the TNF-α, TG, TC and HDL-C in diabetic rats. CoQ_{10} nanoparticles showed a reduction in the CRP levels and curcumin nanoparticles showed reductions in IL-6 as well as CRP in comparison to STZ group which were not significantly different from normal rats. Curcumin showed a strong anti-inflammatory activity by reducing IL-6, TNF-α and CRP, and anti-hyperlipidemic activity by normalising lipid levels in diabetic rats. CoQ_{10} nanoparticles showed anti-inflammatory activity only in terms of CRP and showed antihyperlipidaemic effect by normalising the lipid levels in diabetic rats. The deliverability of CoQ_{10} and curcumin can be improved with the nanoparticulate approach to study the beneficial effects of these agents in oxidative stress and inflammation induced disease states.

5. EVALUATION OF COENZYME Q_{10} AND CURCUMIN NANOPARTICLES IN CHRONIC HYPOXIA INDUCED PULMONARY HYPERTENSION IN THE RAT

5.1. Introduction

Increased numbers of studies are being carried out in identifying the role of oxidative stress in PH. Studies have found that the vascular remodelling associated with the development of PAH in the lamb ductal ligation model is associated with increased production of ROS [Brennan *et al.* 2003]. Evidence also suggests that there is a reciprocal relationship between oxidative stress and ET-1 regulation such that ROS can regulate cellular levels of ET-1 and mediate its secretion [Wedgwood and Black, 2003].

The studies identifying the role of hypoxia in PH involve a wide variety of hypotheses and with isolated cells, isolated pulmonary arterial rings (*in vitro*), perfused lung studies, hypoxic animal models, anesthetized animals and humans, and healthy humans. All the studies in a broader sense report the altered physiology of the pulmonary arteries [Table 5.1]. Hypoxic pulmonary vasoconstriction (HPV) is a physiological phenomenon in which pulmonary arteries constrict in the presence of hypoxia (low oxygen levels) redirecting blood flow to alveoli with higher oxygen content. The small muscular pulmonary arteries are the major sites of HPV. Pulmonary arterioles <70 µm in diameter normally contain only a single elastic lamina without any smooth muscle. However with alveolar hypoxia smooth muscles are extended into these small pulmonary arterioles and the elastic lamina is split into inner and outer layers surrounding the smooth muscles. The extent of muscularisation also increases in the arteries with smooth muscle cells [Anand 1994].

In rats exposed to hypobaric hypoxia, MDA levels in serum, heart, lungs, liver and kidneys were all significantly higher than in control rats by day 21 of exposure indicating increased oxidative stress. The concentration of immunoreactive Mn-SOD in the serum of these rats was raised from day 5 onwards, whereas in liver and lung, it had decreased significantly by day 21. By day 21, GPx activity had increased in the heart and lungs, but decreased significantly in the liver [Nakanishi *et al.* 1995].

Concomitant β-carotene treatment protected the lung parenchyma from the inflammatory reaction and the septal fibrosis, but did not prevent right ventricular hypertrophy and only slightly reduced the thickening of the wall of small arteries and arterioles in MCT induced pulmonary hypertensive rats [Baybutt and Molteni 1999]. Vitamin E has a protective role on the development of PH in MCT treated rats. A modest reduction of the right ventricular hypertrophy in these rats was observed with high-dose vitamin E (3.3 g vitamin E/kg diet) treatment. However, this treatment could not prevent the vascular remodelling caused by MCT [Molteni *et al.* 2004].

A study reveals that the treatment of 20 g/l NAC in drinking water 7 days before and 7 days after the initiation of isobaric hypoxia in a 4 week study, resulted in significant reduction of the right ventricular hypertrophy from 0.510 to 0.430. Their results indicate that the initial (7 days before and 7 days after the initiation of isobaric hypoxia) but not late (last two weeks treatment in 4 weeks of isobaric hypoxic exposure) NAC treatment inhibited hypoxia-induced muscularisation of distal pulmonary arteries [Lachmanova *et al.* 2005].

Table 5.1: Comparison of hypoxic studies from different levels of investigation

Level of Investigation	Changes	Observations
Cellular	Accumulation of HIF1α, Increased ROS generation, Increased intracellular calcium levels, Imbalance of vasoactive substances	Increased cell proliferation of smooth muscle cells and endothelial cells, Smooth muscle cell contraction
Arteries	Altered relaxation, Muscularisation of previously non-muscularised arterioles, and increased medial thickness of other arterioles, Endothelial changes,Adventitial hypertrophy	Vasoconstriction
Lungs	Compromised eNOS activity	Rise in pulmonary artery pressure
Animals	Increased ROS generation & altered antioxidant enzyme levels, Imbalance of vasoactive substances, Altered hemodynamics, Right ventricular hypertrophy	Pulmonary hypertension, Vascular remodelling, Increased oxidative stress, Fatigue
Clinical studies	Increased oxidative stress, Right ventricular hypertrophy, Altered hemodynamics	Pulmonary Hypertension, Shortness of breath, Fatigue, Non-productive cough, Angina pectoris, Fainting or syncope, Peripheral oedema , Rarely haemoptysis

Exposure to hypoxia generates oxidative stress of lung tissue. Studies were conducted based on the hypothesis that oxidative stress may play a role in the hypoxia-induced cardiopulmonary changes [Hoshikawa *et al.* 2001]. In those studies, from 24 h before the initiation of normobaric hypoxia (10%

oxygen) rats were on a dose of 1 % NAC *ad libitum* or allopurinol 50mg/kg at every 12 h for 3 weeks. After 3 weeks the right ventricular hypertrophy was reduced to 0.37 (hypoxic control 0.46) and 0.30 (hypoxic control 0.41) with NAC and allopurinol treatments respectively. Lung tissue levels of phosphatidylcholine hydroperoxide (PCOOH), a primary peroxidation product of phosphatidylcholine, increased from day 1 to day 7 of hypoxic exposure and reached a maximum at day 7 and treatment with NAC and allopurinol inhibited the increase in PCOOH levels in the lungs from the hypoxia-exposed rats. Pro-oxidant enzyme xanthine oxidase (XO) activity in the rat lungs was elevated from day 1 through day 21 of the hypoxic exposure (the maximal values occurred at the third day) and treatment with a XO inhibitor, allopurinol, reduced the increase in PCOOH levels in hypoxia-exposed rat lungs and attenuated PAH, right ventricular hypertrophy, and pulmonary vascular media [Hoshikawa *et al.* 2001]. Hypoxia induced changes like increase in pulmonary artery pressure and right ventricular hypertrophy during the 3 weeks of chronic hypoxia were also significantly attenuated by 21 aminosteroid (U-83836E) and dimethylthiourea [Lai *et al.* 1998].

Another antioxidant, tempol (membrane-permeable superoxide dismutase mimetic), has also been shown to prevent the development of PH in chronic hypoxic rats. Treatment with tempol (86 mg/kg/day in drinking water) normalized right ventricular systolic pressure and reduced right ventricular hypertrophy. The media to lumen diameter ratio of the pulmonary arteries was greater for the hypoxic rats compared to the normoxic rats, and was not reversed by treatment with tempol. However, the results support the possibility that superoxide dismutase mimetics can be useful for the treatment of PH [Elmedal *et al.* 2004].

In vivo and *in vitro* studies demonstrate that erdosteine, a thiol-derived drug, is a potent free radical scavenger. The hypobaric hypoxic rats when treated with this drug the lipid peroxidation levels were reduced and total antioxidant status was increased. The free radical-scavenging properties of erdosteine are the basis of decreased inflammation, protection of pulmonary endothelium and reduced right ventricular hypertrophy in hypobaric hypoxia (0.5 atm) exposed rats after treatment with erdosteine [Uzun *et al.* 2006].

Several antioxidant compounds have been shown to partly but not completely reverse PH and right ventricular hypertrophy. Vascular remodelling is the least affected indication of PH with the antioxidant treatments. However, the antioxidants lowered the lipid peroxidation, inflammation and increased antioxidant status in the experimental PH.

5.1.1. Aim

The objective of this part of the research program is to understand the role of CoQ_{10} and curcumin in ameliorating the PH. The objectives will be achieved by the following specific aims:

- By developing appropriate formulations that can facilitate better peroral delivery with improved efficacy of poorly bioavailable CoQ_{10} and curcumin. In this case, we developed CoQ_{10} and curcumin nanoparticles.
- By evaluating the efficacy of the developed CoQ_{10} and curcumin nanoparticles in suitable animal model by measuring the pharmacological end points such as right ventricular hypertrophy and vascular changes. In this case we have used chronic hypoxic rats.

5.2. Methods

5.2.1. Experimental animals

All procedures on animals were performed according to the United Kingdom Home Office Guide on the Operation of Animals (Scientific Procedures) Act 1986. Animals were housed in the Biological Procedures Unit (BPU), University of Strathclyde, allowed free access to food and water and maintained on a 12 h light and dark cycle.

5.2.1.1. Chronic hypoxic rats

Male Sprague-Dawley rats (180–200 g) were placed in a hypobaric chamber at 600 mbar for two weeks. Age matched controls were housed at atmospheric pressure. The rats were acclimatised for 2 days in the hypoxic chamber during which the pressure was brought down to 600 mbar by reducing 70-75 mbar pressure in each step, and the duration between each step was at least 6 h. After the acclimatisation the chambers were opened everyday to treat the animals. Whenever the chamber is repressurised the pressure was brought up in steps of 70-75 mbar having 10 min interval between them. The chamber was never opened for more than half an hour in a day. After the treatment the chamber was depressurised in 70-75 mbar steps with 10 min interval between each step until 600mbar is reached. The bedding was changed every three days and the food and water were supplied whenever required.

5.2.1.2. Treatment with formulations

The nanoparticles preparations obtained following the procedure described in section 2.2.2.2 of chapter 2. Nanoparticles were centrifuged at 15,000g for 15 min and the supernatant was decanted. The pellet was redispersed in required amount of water to achieve a concentration of 20 mg/ml of drug in the solution. The suspension (20 mg/ml) was prepared in

0.5% w/v sodium carboxy methyl cellulose. All the formulations were freshly prepared on the day of administration. The rats were treated with CoQ_{10} suspension (50 mg/kg) or CoQ_{10} (50 mg/kg) nanoparticles or curcumin (100 mg/kg) nanoparticles or curcumin (100 mg/kg) suspension individually throughout the hypoxic exposure. All the formulations were given orally using a gavage needle. The volumes were less than 1.5 ml per dose. The detailed study plan was shown in table 5.2.

5.2.2. Measurement of right ventricular hypertrophy

After the treatment with different formulations rats were euthanized in a CO_2 chamber. The heart is isolated and the auricles of the heart were separated out. The right ventricle was separated from the left ventricle leaving the septum. The weights of the right ventricle and the left ventricle + septum were then recorded. The ratio between right ventricular weight and left ventricle + septum weights, RV/(LV+S) were used as measures to compare the right ventricular hypertrophy among the treatment groups.

5.2.3. Measurement of haematocrit

Blood was collected immediately from the rats after euthanasia by cardiac puncture in heparinised syringes. The blood was then transferred in to the heparinised capillary tubes. These tubes were spun in a centrifuge at 12,000 rpm for 10 min. Then the lengths of the packed cells and the total length including the plasma were recorded. The haematocrit was expressed as the percentage of the ratio between packed cells to total length of blood.

Table 5.2: Study plan for the chronic hypoxic rats

Group	Acclimatisation		Normoxia														
Normoxic (N)	-	-	¤	¤	¤	¤	¤	¤	¤	¤	¤	¤	¤	¤	¤	¤	
CoQ_{10} nanoparticles (NCN) (50 mg/kg)	-	-	√	-	√	-	√	-	√	-	√	-	√	-	√	-	
	Acclimatisation		Hypoxia														
Hypoxic (H)	-	-	¤	¤	¤	¤	¤	¤	¤	¤	¤	¤	¤	¤	¤	¤	
CoQ_{10} suspension (HCOS) (50 mg/kg)	-	-	√	√	√	√	√	√	√	√	√	√	√	√	√	√	
CoQ_{10} nanoparticles (HCON) (50 mg/kg)	-	-	√	-	√	-	√	-	√	-	√	-	√	-	√	-	Sacrifice
Curcumin suspension (HCUS) (100 mg/kg)	-	-	√	√	√	√	√	√	√	√	√	√	√	√	√	√	
Curcumin nanoparticles (HCUN I) (100 mg/kg)	-	-	√	-	√	-	√	-	√	-	√	-	√	-	√	-	
Curcumin nanoparticles (HCUN II) (100 mg/kg)	-	-	√	√	√	√	√	√	√	√	√	√	√	√	√	√	
Days	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**

¤ - vehicle treated, √ - dose administered

5.2.4. Histology

5.2.4.1. Tissue fixation, processing and paraffin embedding

After euthanasia lungs from the rats were isolated and different lobes were separated. The left lobe was used for histological examination. The lobes were inflated with 10% neutral buffered formalin solution by injecting through the bronchus. Then they were kept in the same solution. These tissues were fixed for at least one day before dehydration. Tissues were dehydrated using the tissue processor (Citadel 1000, Thermo Shandon Ltd, UK), through a series of ethanol and histoclear in the sequence presented in table 5.3. Tissues were then embedded in paraffin wax using a tissue embedder (EG1140H, Leica, Germany).

Table 5.3: Wax embedding

Solution	Duration
100% ethanol	2 h
100% ethanol	2 h
50:50 ethanol:histoclear	1 h
100% histoclear	1 h
100% histoclear	1 h
Paraffin wax	2 h
Paraffin wax	2 h

5.2.4.2. Section cutting, slide preparation and staining

Paraffin blocks were cut into 3 µm sections with a rotary microtome (RM 2125 RTF, Leica, Germany). Sections were floated on the surface of the water bath at around 50°C and mounted onto uncoated glass slides for staining. Slides were then heated in an oven at 60°C for 1 h to remove excess paraffin wax and stored at room temperature.

Table 5.4: Staining procedure

Step	Solution	Duration
1	Histoclear	1 min
2	Histoclear	1 min
3	Histoclear	1 min
4	100% ethanol	1 min
5	100% ethanol	1 min
6	Distilled water	1 min
7	Haematoxylin Gill III	2 min
8	Distilled water	1 min
9	1% acid alcohol	1 min
10	Distilled water	1 min
11	Scott's tap water substitute	2 min
12	Distilled water	1 min
13	3% eosin	1 min
14	100% ethanol	1 min
15	100% ethanol	1 min
16	100% ethanol	1 min
17	Histoclear	1 min
18	Histoclear	1 min
19	Histoclear	1 min
20	Histoclear	1 min

Slides were stained for H&E in an auto-staining processor (VaristainnV24-4, Thermo Shandon, UK). The H&E staining programme is depicted in table 5.4. Then the sections were mounted using DPX mountant and cover slips.

5.2.5. Immunohistology

5.2.5.1. Tissue fixation, processing and paraffin embedding

After euthanasia lungs from the rats were isolated and different lobes were separated. The left lobe was used for histological examination. The lobes were inflated with 10% neutral buffered formalin solution by injecting through the bronchus. Then they were kept in the same solution. These

tissues were fixed for at least one day before dehydration. Tissues were dehydrated and embedded in wax the next day or within next two days using the tissue processor (Citadel 1000, Thermo Shandon Ltd, UK), through a series of ethanol and histoclear in the following sequence given in table 5.5. Tissues were then embedded in paraffin wax using a tissue embedder (EG1140H, Leica, Germany).

Table 5.5. Dehydration and paraffin embedding

Solution	Duration
70% ethanol	2 h
90% ethanol	2 h
100% ethanol	2 h
50:50 ethanol:histoclear	1 h
100% histoclear	1 h
100% histoclear	1 h
Paraffin wax	2 h
Paraffin wax	2 h

5.2.5.2. Silanation of slides

Slides were silanated to improve tissue adhesion to the slide. Slides were arranged in racks and they were immersed in 100% acetone for 10 min followed by air-drying for 10 min. Then the slides were immersed in solution of 5% of 3-aminopropyltriethoxysilane (APES/silane) in acetone for 10 minutes. They were then gently rinsed in a water bath with running water in order to wash off excess silane. They were then immediately covered in tissue paper to prevent dust particles from attaching to the slides. The slides were left completely covered for three days.

5.2.5.3. Section cutting and slide preparation

Paraffin blocks were cut into 3 µm sections with a rotary microtome (RM 2125 RTF, Leica, Germany). Sections were floated on the surface of the water bath at around 50°C and mounted onto silanated slides for staining.

Slides were then heated in an oven at 60°C for 1 h to remove excess paraffin wax and stored at room temperature.

5.2.5.4. Staining protocol

The de-paraffinized slides were rehydrated in histoclear to alcohol using the auto stainer (three histoclear washes for 5 min each followed by two alcohol washes for 5 min each and distilled water wash for 5 min). They were then put in 0.3% H_2O_2 for 10 min. This treatment blocks the endogenous peroxidise activity. The slides were gently rinsed in phosphate buffered saline (PBS) for 5 minutes. This was followed by high temperature microwave pre-treatment in order to expose the antigen that was masked in the tissue processing. Tris EDTA (ethylenediaminetetra acetic acid) solution was pre-warmed in microwave for 13 min at 900 W. The slides were then placed in this boiling solution and microwaved for a further 7 min in the microwave pressure cooker. Then the cooker was depressurised and opened. The slides were left to cool down for 20 min and then were rinsed in cold tap water bath for 5 min followed by two phosphate buffered saline rinses for 2.5 min each. Slides were then allowed to air dry and encircled with a wax pen. 20% normal goat serum (NGS) in PBS was applied to the sections and was incubated for 20 min. Treatment with NGS reduces the non-specific binding of the secondary antibody and subsequent background staining. The NGS was then removed from the sections and the primary antibody was applied and the slides were further incubated for 1 h at room temperature. The anti-smooth muscle α-actin mouse monoclonal (1:1000) was applied to the sections. Then the sections were washed 2 times with PBS for 2.5 min each. Horse radish peroxidise labelled secondary antibody (goat anti-mouse antibody 1:400) was added to the sections and incubated for 30 min. Following the incubation slides were washed twice with PBS for 2.5 min each

time. Then activated 3,3-diaminobenzidinetetrahydrochloride (DAB) was added and incubated for 10 min. After this DAB solution was removed and 1% copper sulphate solution was added and allowed for 5 min. Finally the slides were rinsed with distilled water for 5 min. The slides were counterstained and dehydrated in the auto-stainer following the program given in table 5.6. Finally sections were mounted in DPX mountant medium and coverslips were placed on top of the tissue sections.

Table 5.6. Counterstaining sequence

1	Haematoxylin Gill III	15 sec
2	Distilled water	1 min
3	1% acid alcohol	1 min
4	Distilled water	1 min
5	Scott's Tap water substitute	2 min
6	Distilled water	1 min
7	100% ethanol	2 min
8	100% ethanol	2 min
9	100% ethanol	2 min
10	Histoclear	2 min
11	Histoclear	2 min
12	Histoclear	2 min
13	Histoclear	2 min

5.2.5.5. Slide analysis and scoring

Actin staining was used as a measure of investigating the extent of muscularization of arteries. There are several previously reported methods to quantify and differentiate the extent of muscularization of the small pulmonary arteries in chronically hypoxic from normoxic animals. They include the measurement of the arterial wall such as the thickness of smooth muscle cells in micrometers, which is a highly labour-intensive method, calculating the percentage of vessels with double elastic lamina, and staining of smooth muscle cells with actin and measuring the intensity of the stain to

quantify the extent of thickening of the arteries in both normoxic and chronically hypoxic rats. In the current study 50 consecutive small pulmonary arteries less than 100 µm were photographed from each slide using 800x magnification. Vessels were counted as thickened if they possessed both of the following criteria. 1. The artery wall thickness was at least one-third the radius on any given side of artery wall and 2. At least 50% of the total wall area was thickened. Any vessels, which were on the borderline, were not counted or taken into account and number of discounted vessels was negligible. The number of thickened arteries was expressed as percentage of the total arteries less than 100 µm taken into consideration.

5.2.6. Tissue distribution studies

5.2.6.1. *In vivo* analytical method for curcumin detection

The concentration of curcumin in plasma and tissue samples was analysed using HPLC on a C18 column (Hypersil GOLD 15 cm x 4.6 mm, 5 µm) using mobile phase consisting of methanol, acetonitrile and 2.8% acetic acid (35:30:35). Estradiol was used as internal standard. The HPLC system consisted of ChromQuest acquisition software, autosampler connected with LC Pump and PDA detector (Thermo Finnigan Surveyor System). Estradiol was detected at 280 nm and curcumin at 425 nm.

Curcumin extraction was carried out by taking plasma/tissue homogenate samples (100 µl) in micro-centrifuge tubes to which 20 µl of ethyl acetate containing 500 µg/ml of estradiol (internal standard) was added. To precipitate the proteins 100 µl of 2.8% acetic acid was added and vortexed. To the above mixture 500 µl of ethyl acetate was added and vortexed again. Extract was separated and vacuum dried and reconstituted in 125 µl of acetonitrile. 100 µl of this solution was injected to the HPLC. The

calibration curve was plotted from the ratio of peak area values, of curcumin to estradiol, obtained from HPLC using 100 µl plasma spiked with curcumin standards (20, 50, 100, 200 and 500 ng/ml) and following the similar treatment of the samples as in the case of plasma or tissue homogenates. The method was validated for accuracy and precision.

5.2.6.2. Curcumin tissue distribution in PH rats

PH rat groups CUS, CUN I and CUN II at the end of the treatment period were euthanised, blood was collected and brain, heart, kidneys, liver, lungs and spleen were isolated. Plasma was separated from the blood and the tissues were homogenised in pH 7.4 buffer followed by curcumin extraction and analysis using HPLC.

5.2.6.3. Curcumin tissue distribution in hypoxic and normoxic rats

Male Sprague Dawley rats weighing 180-200 g were housed in hypoxic chamber for acclimatisation for 2 days, during which the pressure was brought down to 600 mbar by reducing 70-75 mbar pressure in each step, and the duration between each step was at least 6 h. Six rats were devided into two groups, one group was administered 100 mg/kg curcumin suspension and the other was administered 100 mg/kg curcumin nanoparticles. After 24 h rats were euthanised, blood was collected and brain, heart, kidneys, liver, lungs, spleen, stomach, small intestine, ceacum and large intestine were isolated. Plasma was separated from the blood and the tissues were homogenised in pH 7.4 buffer followed by curcumin extraction and analysis using HPLC.

To study the curcumin tissue distribution in normoxic conditions after 2 h and 24 h, twelve male Sprague Dawley rats weighing 180-200 g were divided into four groups. Two groups received curcumin suspension and the other two groups received curcumin nanoparticles each 100 mg/kg. After 2 h

and 24 h one group receiving suspension and one group receiving nanoparticles were euthanised, blood was collected and brain, heart, kidneys, liver, lungs, spleen, stomach, small intestine, ceacum and large intestine were isolated. Plasma was separated from the blood and the tissues were homogenised in pH 7.4 buffer followed by curcumin extraction and analysis using HPLC.

5.3. Results

5.3.1. Pulmonary hypertension in chronic hypoxic rats

There was a significant weight loss with the hypoxic treatment after 2 weeks [Fig 5.1]. Hypoxic exposure resulted in increased haematocrit and right ventricular hypertrophy [Fig. 5.2 & Fig. 5.3]. In the histological examination using H&E stained slides [Fig. 5.4] and smooth muscle α-actin stained slides it is obvious that the small pulmonary arterial walls were thickened [Fig. 5.5].

All the treatments have been ineffective in preventing the loss of body weight [Fig. 5.1] increase in the haematocrit percentage [Fig. 5.2] and the right ventricular hypertrophy [Fig. 5.3]. The right ventricular hypertrophy results were corroborated with the histological examination of the pulmonary arterial wall thickness [Fig 5.4 & 5.5]. Scoring of the smooth muscle α-actin immunostained slides also suggests the ineffectiveness of treatments in reducing the vascular remodelling [Table 5.7]. The results indicate that there is no significant improvement in the nanoparticles and the suspension treated groups in comparison to the hypoxic group. The CoQ_{10} nanoparticles treated normoxic rats (NCN) show similar parameters to the normoxic control rats (N) suggesting no effect of nanoparticles on the lungs in normoxia. Figure 5.6 shows the presence of curcumin nanoparticles as

clusters, probably in the alveolar macrophages, in the lung sections of the CUN II treated rats stained with H&E at the end of the treatment period.

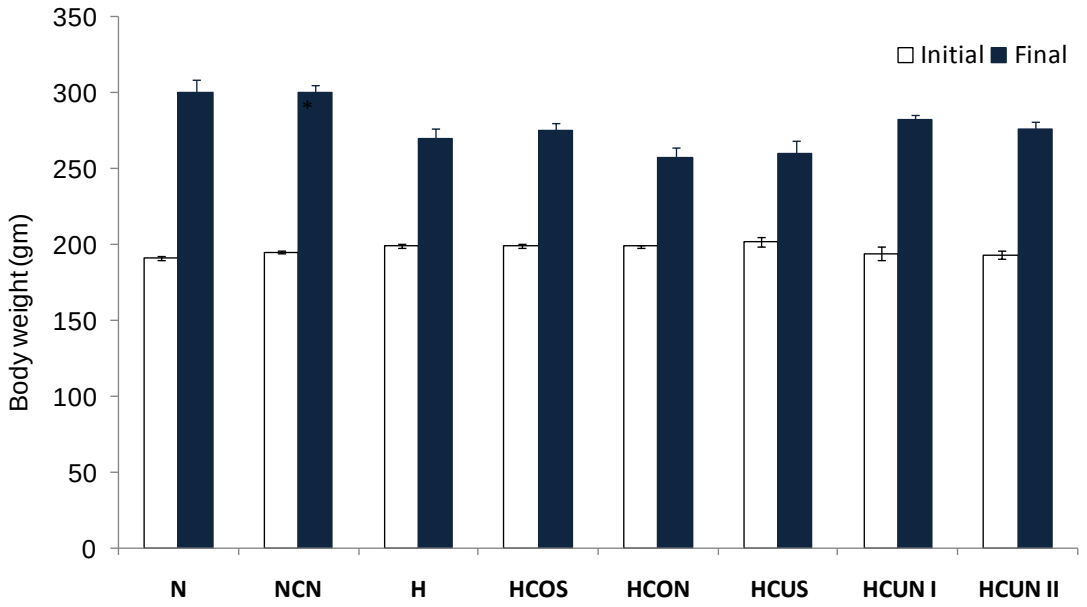


Figure 5.1. Initial and final body weights of different groups of rats. Unfilled bars represent initial body weights at the start of the experiment and filled bars represent the final body weights at the end of the experiment. N- normoxic, NCN- normoxic CoQ_{10} nanoparticles treated (once in two days), H- hypoxic, HCOS – hypoxic CoQ_{10} suspension treated (daily), HCON - hypoxic CoQ_{10} nanoparticles treated (once in two days), HCUS - hypoxic curcumin suspension treated (daily), HCUN I – hypoxic curcumin nanoparticles treated (once in two days) and HCUN II - hypoxic curcumin nanoparticles treated (daily) groups. Values are expressed as mean ± S.E.M. (n=6-10). Hypoxic rats showed statistically significant body weight loss in comparison to the normoxic rats. Treatment with CoQ_{10} and curcumin suspensions and nanoparticles in hypoxic groups showed comparable results to the hypoxic group.

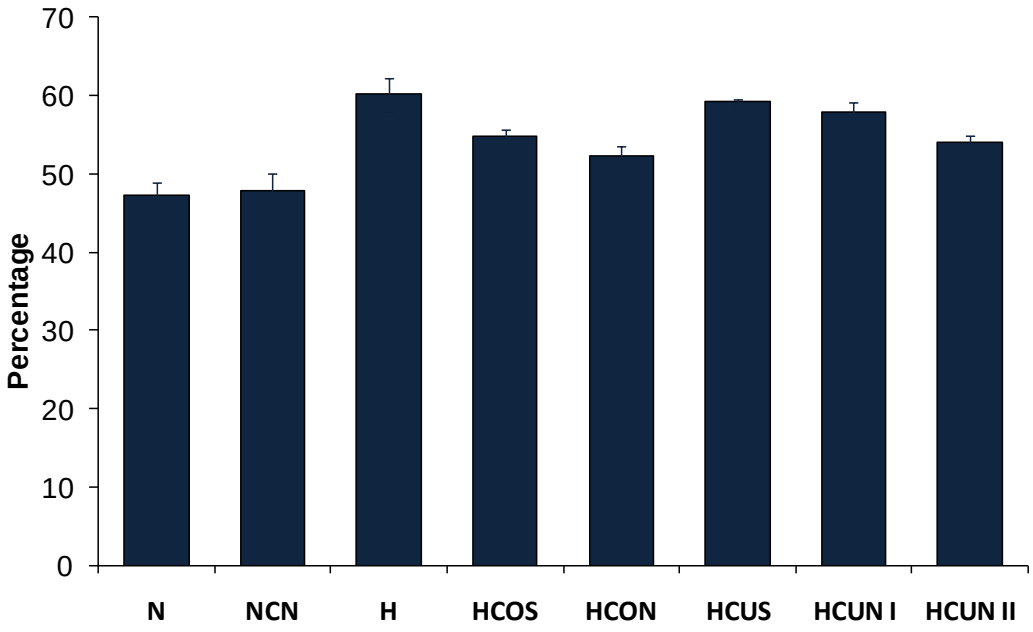


Figure 5.2. Haematocrit percentage in hypoxic rats. N- normoxic, NCN- normoxic CoQ_{10} nanoparticles treated (once in two days), H- hypoxic, HCOS – hypoxic CoQ_{10} suspension treated (daily), HCON - hypoxic CoQ_{10} nanoparticles treated (once in two days), HCUS - hypoxic curcumin suspension treated (daily), HCUN I – hypoxic curcumin nanoparticles treated (once in two days) and HCUN II - hypoxic curcumin nanoparticles treated (daily) groups. Values are expressed as mean ± S.E.M. (n=6-10). Hypoxia increased haematocrit percentage significantly in comparison to normoxic rats. Treatment with CoQ_{10} and curcumin suspensions and nanoparticles in hypoxic groups showed comparable results to the hypoxic group.

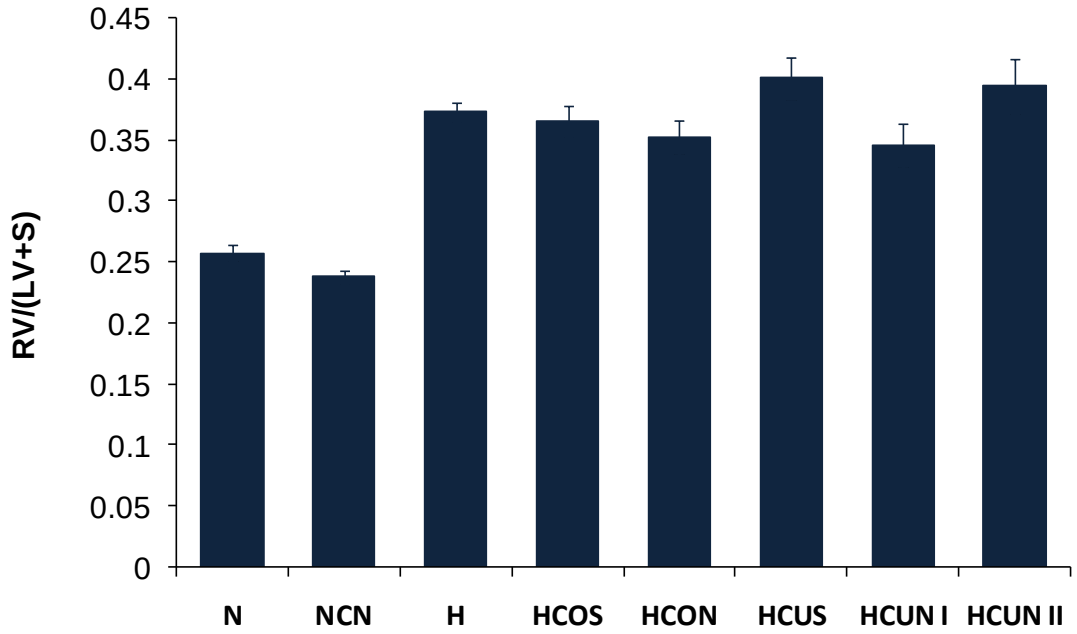


Figure 5.3. Right ventricular hypertrophy (RV/LV+S) in hypoxic rats. N- normoxic, NCN- normoxic CoQ_{10} nanoparticles treated (once in two days), H- hypoxic, HCOS – hypoxic CoQ_{10} suspension treated (daily), HCON - hypoxic CoQ_{10} nanoparticles treated (once in two days), HCUS - hypoxic curcumin suspension treated (daily), HCUN I – hypoxic curcumin nanoparticles treated (once in two days) and HCUN II - hypoxic curcumin nanoparticles treated (daily) groups. Values are expressed as mean ± S.E.M. (n=6-10). Hypoxia increased right ventricular hypertrophy significantly in comparison to normoxic rats. Treatment with CoQ_{10} and curcumin suspensions and nanoparticles in hypoxic groups showed comparable results to the hypoxic group.

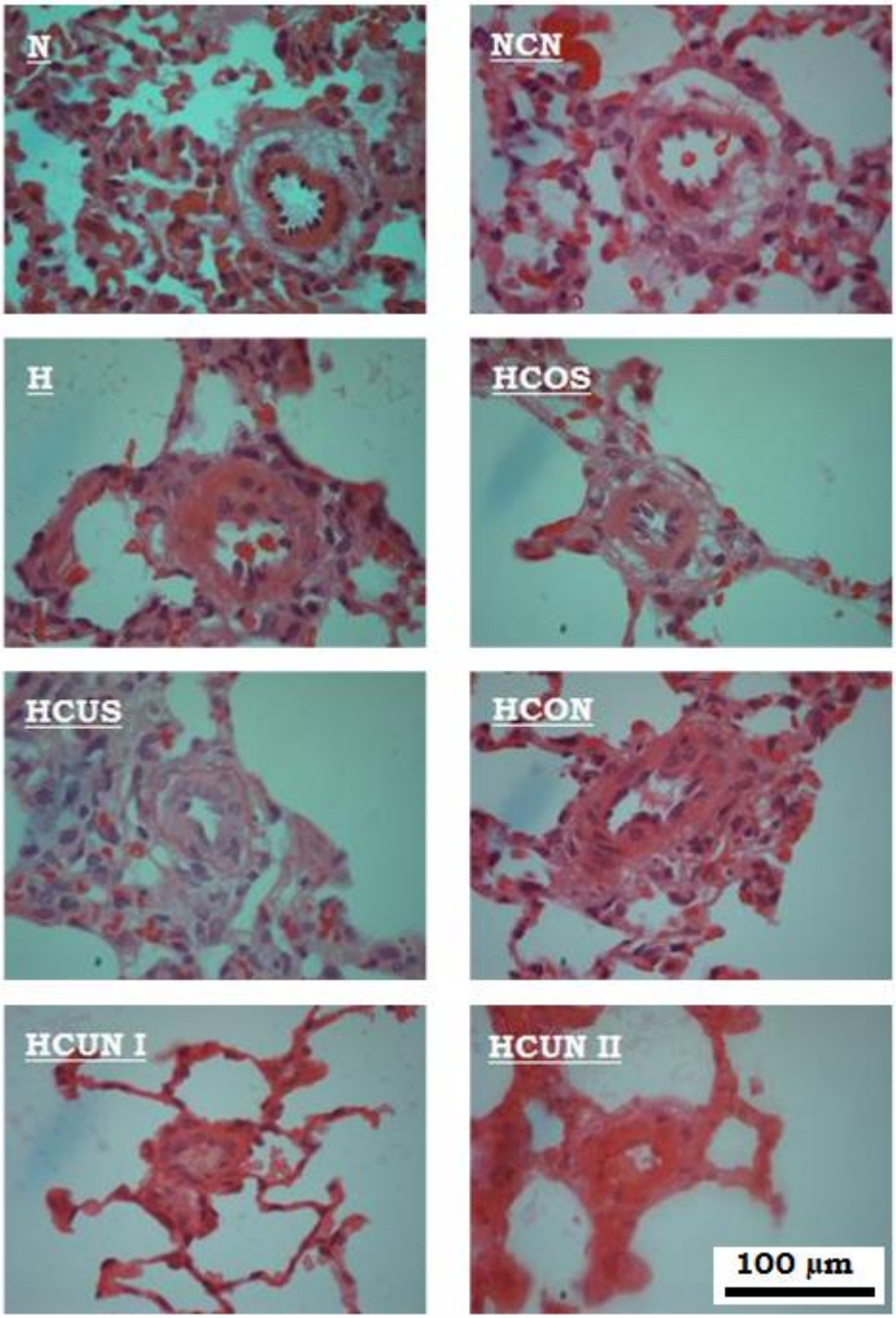


Figure 5.4: Haematoxylin and eosin (H&E) stained histological photographs of lung sections obtained after different treatments. The small pulmonary arterioles are thickened in case of hypoxic treatment in comparison to normoxic rats and the different antioxidant treatments have no effects on the hypoxia induced vascular hypertrophy. (All the pictures were taken at 40x2x10 magnification)

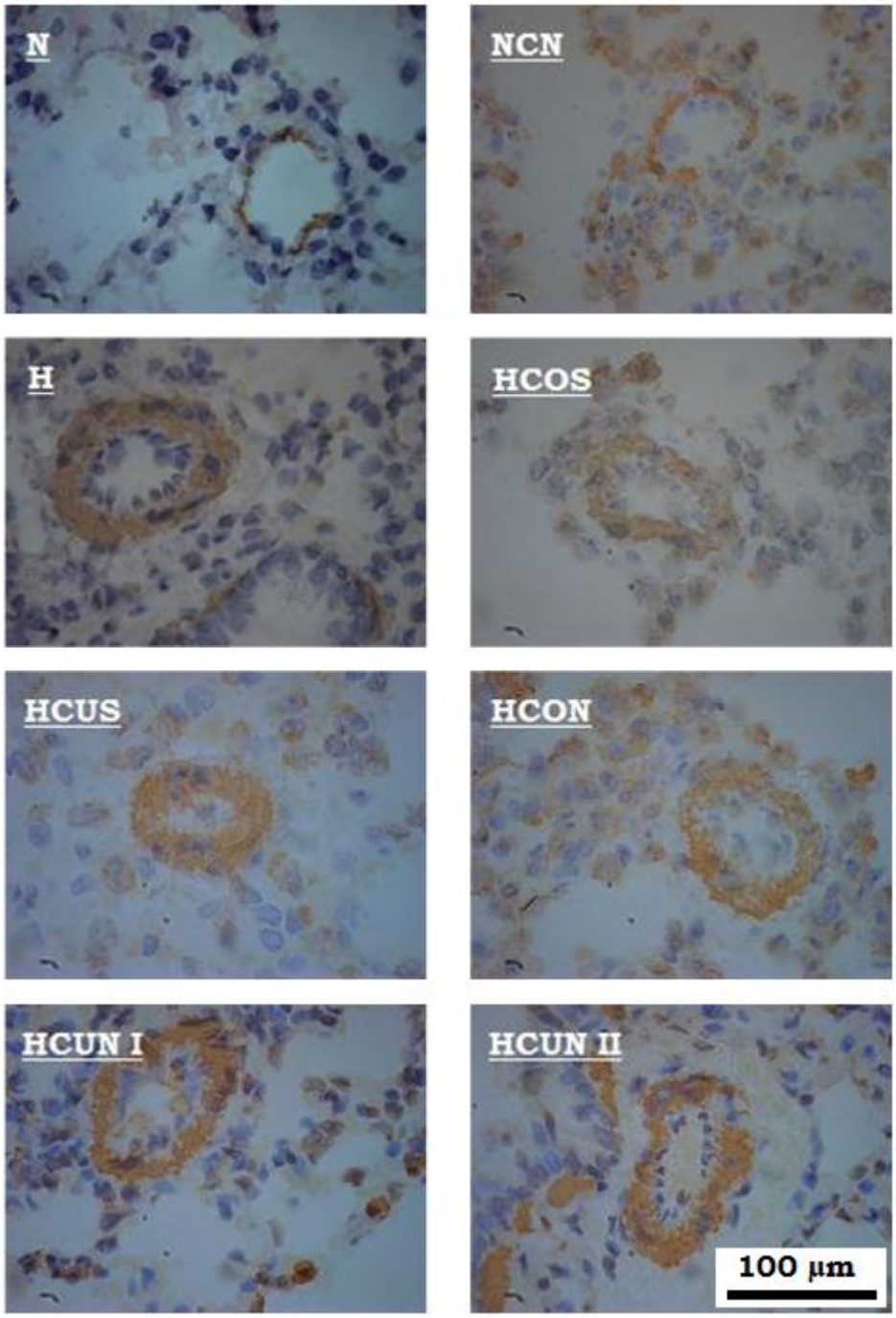


Figure 5.5. Immunohistological photographs of lung sections, for smooth muscle α-actin, obtained after different treatments. Brown colouration depicts the presence of smooth muscle α-actin and was very thick in the hypoxic lungs in comparison to normoxic lungs (All the pictures were taken at 40x2x10 magnification).

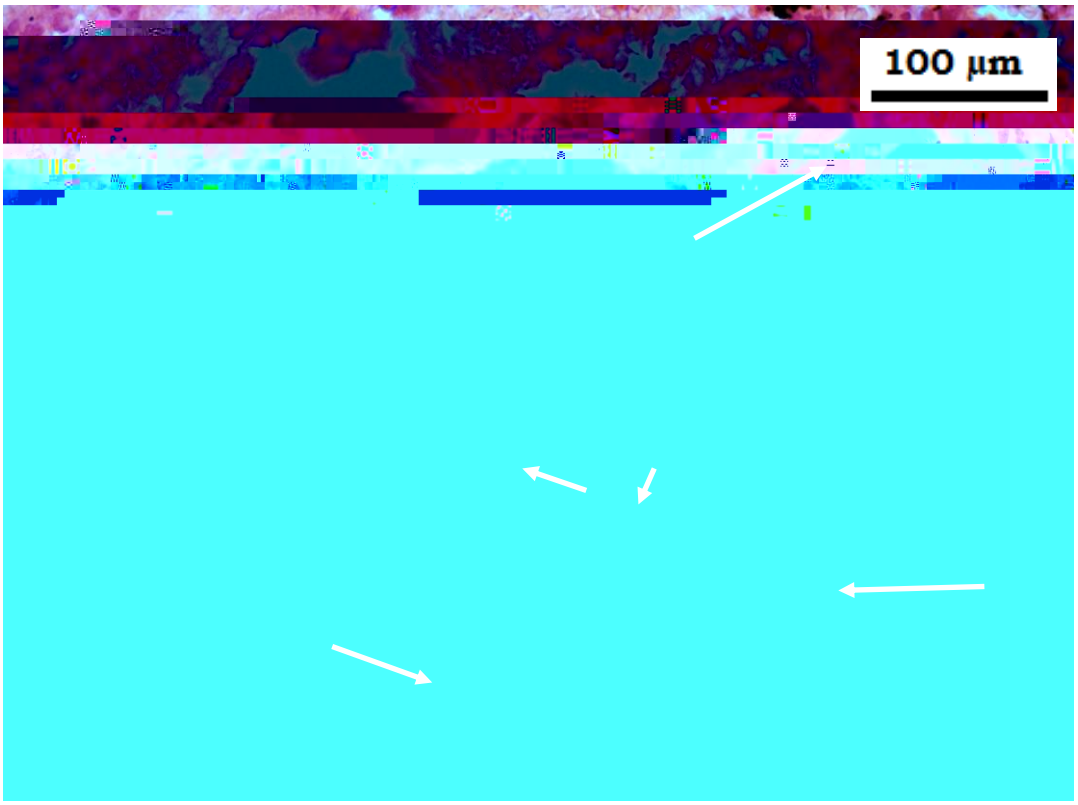


Figure 5. 6. Haematoxylin and eosin stained lung section of the rat treated with curcumin nanoparticles daily (HCUN II). The section shows accumulation of nanoparticles in the lung outside the vasculature probably in the macrophages is observed due to the inherent yellow colour of curcumin as yellowish dark spots. (400x)

Table 5.7. Scoring of slides immunostained for smooth muscle α-actin

Group	Percentage of thickened arteries
N	**6±4**
NCN	**6±3**
H	**39±16**
HCOS	**55±9**
HCON	**59±11**
HCUS	**60±10**
HCUN I	**55±7**
HCUN II	**32±10**

Values are expressed as mean±Std; n=6

H is significantly different from N and HCOS, HCON, HCUS, HCUN I & HCUN II are comparable to H.

5.3.2. Tissue distribution in PH, hypoxic and normoxic rats

HCUS group showed curcumin levels in lungs and spleen but no detectable levels were present in plasma, brain, heart, kidneys and liver. HCUN I group showed curcumin levels only in spleen. HCUN II group showed curcumin levels in heart, kidneys, lungs and spleen [Table 5.8].

Table 5.8. Tissue distribution of curcumin in PH rats (n=6)

Group	*Plasma*	*Brain*	*Heart*	*Kidneys*	*Liver*	*Lungs*	*Spleen*
HCUS	ND	ND	ND	ND	ND	48±12	60±4
HCUN I	ND	ND	ND	ND	ND	ND	50±10
HCUN II	ND	ND	26±2	47±11	ND	42±10	72±13

Units for plasma levels are ng/ml and for tissues ng/gm; n=6
ND = not detectable; limit of detection = 20 ng/ml

Table 5.9. Plasma and tissue concentrations of curcumin in normoxic and hypoxic rats (n=3)

Time (h)	Formulation	Hypoxia	Plasma	Brain	Heart	Kidneys	Liver	Lungs	Spleen	Stomach	Ceacum	Small Intestine	Large Intestine
2	Suspension	no	ND	ND	ND	ND	ND	ND	1519±992	ND	ND	1198±225	ND
24	Suspension	no	ND	ND	ND	ND	ND	191±13	ND	3607±61	7487±1184	2290±741	1796±585
2	Nanoparticles	no	182±44	ND	43±11	ND	ND	46±5	137±20	188±57	ND	841±27	ND
24	Nanoparticles	no	ND	274±45	ND	ND	ND	252±11	ND	439±199	750±16	72±44	433±52
24	Suspension	yes	ND	ND	ND	77±6	ND	ND	ND	ND	ND	222±15	301±82
24	Nanoparticles	yes	ND	ND	ND	255±35	ND	57±14*	25±2	93±11*	ND	92±11	51±16*

Both the formulations were administered orally at 100 mg/kg dose; n=3

Units for plasma levels are ng/ml and for tissues ng/gm.

ND = not detectable; limit of detection = 20 ng/ml

* vs 24h normoxic nanoparticles treated group

Curcumin was seen in the plasma of nanoparticles treated group after 2 h. Plasma of curcumin suspension treated rats either normoxic or hypoxic showed no detectable levels of curcumin after 2 h and 24 h [Table 5.9]. Curcumin was not detected in the livers of all the rats administered suspension or nanoparticles. At 24 h nanoparticles treated normoxic rats showed presense of curcumin in brain but not hypoxic rats. At 2 h nanoparticles showed detectable levels of curcumin in heart. Only hypoxic rats showed the presense of curcumin in kidneys. Lung levels of curcumin were detected in all the nanoparticles administered groups. In the lungs of suspension administered rats after 24 h curcumin was detected and in hypoxic rats it is not detected. Lung levels of curcumin in rats treated with curcumin nanoparticles decreased significantly in hypoxic rats in comparison to the normoxic rats.

After 24h of oral administration of suspension or nanoparticles, curcumin was seen in the stomach, caecum, small intestine and large intestine of the normoxic rats. At 2 h suspension showed levels in small intestine while nanoparticles showed levels in stomach and small intestine. In hypoxic rats curcumin was detected in small and large intestines of the suspension and additionally in stomach with nanoparticles treated groups.

5.4. Discussion

In rats PH due to chronic hypoxia develops during the first two weeks of exposure to hypoxia and then it stabilises and does not increase in severity [Lachmanova *et al.* 2005]. There has been growing literature on the free radical injury to pulmonary vascular wall in the early days of the hypoxic exposure [Hoshikawa *et al.* 2001, Lachmanova *et al.* 2005, Elmedal *et al.* 2004, Uzun *et al.* 2006]. Antioxidant treatment just before and at the beginning of hypoxia is thought to be more effective in reducing pulmonary hypertension.

The rats in hypoxic chambers had decreased body weights in comparison to the normoxic rats. The body weight gain in hypoxic rats was decreased at the acclimatisation phase and later on started to follow the same trend as normoxic rats, however, at the end of the experiment, the hypoxic rats were less in weight in comparison to normoxic rats. There was no effect of CoQ_{10} and curcumin treatments either in the form of suspension or in the form of nanoparticles in comparison to the untreated hypoxic rats.

The right ventricular hypertrophy was seen in hypoxic rats after 2 weeks exposure to hypobaric hypoxia. The effect of different treatments did not show any effect on the RV/(LV+S). Many of the antioxidants have shown positive effects on right ventricular hypertrophy which include vitamin E [Molteni *et al.* 2004], NAC [Lachmanova *et al.* 2005], tempol [Elemdal *et al.* 2004], and allopurinol [Hoshikawa *et al.* 2001]. The mechanisms of actions of all these antioxidants might be different. Vitamin E is a lipophilic antioxidant which acts by reacting with the lipid peroxides formed in lipid peroxidation chain reaction, NAC is a precursor of the antioxidant glutathione, tempol is superoxide dismutase mimetic and allopurinol is the inhibitor of xanthine oxidase which is responsible for generation of oxidative stress. Although these agents have different mechanisms of actions they have been shown to be effective partly in reducing the right ventricular hypertrophy. The common phenomenon associated with all these antioxidants is all of them are associated with endogenous antioxidants like glutathione, superoxide dismutase or redox network which operates endogenously. Apart from the mechanism of action other parameters might also have a role in inactivity of the CoQ_{10} and curcumin. NAC, tempol, allopurinol except vitamin E are hydrophilic and have no known bioavailability problems associated with them, on the other hand CoQ_{10} and curcumin are poorly bioavailable. The

bioavailability of CoQ_{10} and curcumin is less than 10%, which is contributing to their under utilisation.

In the present study nanoparticulate formulations of CoQ_{10} and curcumin have shown no improvements in decreasing the right ventricular hypertrophy. CoQ_{10} and curcumin nanoparticles showed positive effects in lowering the inflammatory cytokines in the STZ induced diabetes, where the effect is directly correlated to the pharmacological properties of the molecules studied. In case of chronic hypoxic rats the development of right ventricular hypertrophy and vascular remodelling involves multiple pathways and is poorly understood.

The haematocrit percentage increased in hypoxic animals. There was no effect with both CoQ_{10} and curcumin nanoparticles and suspension treatment on haematocrit. The histological examination of hypoxic rats shows vascular hypertrophy characterised by reduced lumen thickness and increased vessel wall thickness of small pulmonary arteries. The treatments with different antioxidant formulations have shown no improvements in qualitative analysis of the histological sections of the lungs. The detailed quantitative analysis suggests that with hypoxia muscularisation of small pulmonary arteries thickens the vessel wall and there is no significant effect on the wall thickness with different antioxidant treatments [Table 5.7].

The tissue distribution of curcumin in HCUS and HCUN II groups showed that curcumin reached lungs with daily suspension and nanoparticles treatment (HCUS 48±12 ng/mg, HCUN II 42±10 ng/mg). In HCUN II rat lungs curcumin nanoparticles were observed in histological examination as clusters probably in the alveolar macrophages [Fig. 5.6], which also confirms that with nanoparticles treatment curcumin reached the lungs. However, the distribution of curcumin nanoparticles was

predominantly extravascular but in PH the main source of oxidative stress is vascular wall. Probably this could be the reason for the inactivity of the curcumin in PH.

The inactivity of the curcumin in PH also prompted us to investigate the differences in distribution of curcumin delivered in suspension and nanoparticulate form under hypoxic and normoxic conditions. Hypoxia causes increase in aortic blood flow and alters tissue blood flows [Kuwahira *et al.* 1993]. Differences in blood flow due to hypoxia have been reported to alter the pharmacokinetics of some pharmaceutical compounds [Souich *et al.* 1992, Souich *et al.* 1982]. In the present experiment we observed decreased levels of curcumin in the lungs and increased levels in the kidneys of hypoxic rats receiving suspension as well as nanoparticles. Decreased levels of curcumin in hypoxic lungs might have also contributed to the inactivity of curcumin in experimental PH developed with chronic hypoxia.

It is also observed that the curcumin nanoparticles delivered drug to the brain when given orally. A study by a different group showed that curcumin nanoparticles crossed the blood brain barrier and localised in specific sites in the brain [Chiu *et al.* 2011]. Curcumin is found mainly in the intestinal tract after oral administration which is evident from considerable high levels in stomach, ceacum, small intestine and large intestinal tissue. The levels in the lower part of the intestine i.e., ceacum and large intestine increase with time from 2 h to 24 h. There is a difference seen in the accumulation of curcumin from suspension and nanoparticles in the intestinal tissues. We have not seen detectable amounts of curcumin in some tissues with some treatments. In other studies where sensitive analytical methods (Ultraperformance liquid chromatography) were used ~4 ng/mg levels of curcumin were detected in the liver of rats treated with 340 mg/kg

curcumin [Marczylo *et al.* 2009]. HPLC method is not that sensitive (with limit of detection 20 ng/ml) and the dose we used is only 100 mg/kg, thus, we have not seen any levels in liver and similar is the case with other tissue levels. The nanoparticles seem to have increased the distribution of curcumin into major organs when compared to the suspension of curcumin.

5.5. Conclusion

Although oxidative stress has been implicated in the pathogenesis of PH it might not be the primary cause. There were mixed results on the effect of antioxidants in PH. In PH many antioxidants have shown to reduce the inflammatory and oxidative stress related effects and showed modest effects on the right ventricular hypertrophy and vascular remodelling which are the serious symptoms of the PH. Thorough understanding of the role of oxidative stress is still needed in the pathogenesis of PH. The antioxidants CoQ_{10} and curcumin are not effective in reducing the right ventricular hypertrophy and vascular remodelling in chronic hypoxic rats; similar is the case with the nanoparticles formulations of the both. Nanoparticles are known to improve the bioavailability and in turn therapeutic efficacy, failure of nanoparticles formulation suggests the ineffectiveness of the antioxidants CoQ_{10} and curcumin in chronic hypoxic rats when given orally.

6. STUDY OF ABSORPTION OF NANOPARTICLES

6.1. Introduction

Nanoparticulate delivery systems are emerging as promising drug delivery systems in many contexts. They can be used to deliver small/large or soluble/insoluble molecules by practically every possible route of administration, offering potential benefits that can not be realised by the conventional forms [Peek *et al.* 2008; Vasir *et al.* 2007; Sengupta *et al.* 2007]. In each case, it is important to be able to know the fate of nanoparticles and the active ingredients in the nanoparticles, to determine their final distribution in the target tissue. The uptake of nanoparticles is size dependent, the smaller the size the better is the uptake [Desai *et al.* 1996]. Nanoparticles prepared using hydrophobic polymers like PLGA are more stable in the intestine and are supposed to be taken up intact predominantly, the particles prepared with hydrophilic polymers might distort before absorption. Apart from size and composition, surface characteristics and architecture also influence the absorption of the nanoparticles. The same characteristics also influence the kinetics of the nanoparticles and in turn the kinetics of the loaded active ingredient. Nanoparticles are generally taken up by the reticuloendothelial system which results in decreased circulation times. Polyethylene glycol coating and small particle size are believed to decrease the uptake by the reticuloendothelial system [Plapied *et al.* 2011]. Most studies focus on the evaluation of the kinetics of the active ingredient, therapeutic efficacy or immune response, but the fate of nanoparticles and the polymers is poorly understood.

The techniques most often employed in nanoparticle tracking include gamma scintigraphy, fluorescence or electron microscopy. These techniques require radio-labelling, fluorescence tagging or loading of an electron dense

agent onto the nanoparticles, respectively [Rolland *et al.* 1988; Becker *et al.* 2007; Panagi *et al.* 2001; Sun *et al.* 2007; Panyam *et al.* 2003]. Although these techniques provide useful insights in the study of absorption and distribution of the nanoparticles they are cumbersome to perform and require high end equipment. The use of simple histological stains and light microscopy can make the studies related to absorption and distribution of nanoparticles easier and faster. However, a tracer which can give colour in the histological examination is needed. Use of enzymes which can give coloured products from substrates might be useful for this purpose. One such enzyme is β-galactosidase (β-gal), which has been widely used as a reporter gene product and marker enzyme [Lampson *et al.* 1993]. Commonly used substrates include X-gal and ONPG [Romanos *et al.* 1992; Hung *et al.* 2001]. The X-gal substrate gives a colour reaction product that is readily visualized by light microscopy [Romanos *et al.* 1992; Lampson *et al.* 1993]. Entrapping the enzyme β-gal into the nanoparticles and using the X-gal substrate to track the nanoparticles seems to be a plausible approach and attempted in this research program.

Nanoparticles using preformed polymers are generally prepared using emulsion techniques which often use harsh conditions. Enzymes are highly sensitive to temperature, pH and ionic strength and β-gal is no exception. The stability of β-gal at 37°C decreases markedly with increase in the pH from 5.9 to 8.0 and increased ionic strength in solution gives greater stability [Heyworth *et al.* 1981]. Other enzymes were encapsulated in the nanoparticles before by use of freeze-thaw encapsulation process [Dziubla 2005] or use of different type of polymer which facilitates the release of enzyme in its active form. Therefore, the first step was to develop a method for entrapment of β-gal in nanoparticles, in a way that preserves enzyme

activity during processing. The optimization process was started with a simple procedure routinely applied in preparation of nanoparticles [Bala *et al.* 2005]. However, this process resulted in nanoparticles without any activity of the β-gal, which was checked using ONPG or X-gal assay.

6.1.1. Aim

The objective of this part of the research program is to understand the absorption mechanisms of the nanoparticles through the oral route. The objectives will be achieved by the following specific aims:

- By developing appropriate formulations that can facilitate identification of the nanoparticles in tissues after oral administration. In this case, we developed β-gal loaded nanoparticles.
- By evaluating the efficacy of the developed β-gal loaded nanoparticles' in retaining the β-gal activity *in vitro* and *in vivo*. For *in vivo* tracking of β-gal nanoparticles, these particles were administered orally to rats and histological sections were examined.

6.2. Materials and methods

6.2.1. Materials

β-gal loaded nanoparticles were prepared following the procedure described in section 2.2.2.3 of chapter 2. ONPG, X-gal, dimethyl formamide (DMF), nuclear fast red and aluminium sulphate were purchased from Sigma.

6.2.2. Size and zeta potential measurement

The nanoparticles were characterized using a zeta sizer (Nano ZS, Malvern Instruments, Malvern, UK) for size (average of 5 measurements for one batch) and zeta potential (average of 20 measurements for a batch). The

size measurements were carried out in the buffer used for the preparation of nanoparticles and in the reacton medium after incubation with the X-gal.

6.2.3. Staining of β-gal loaded nanoparticles

β-gal nanoparticles were stained using 2 different substrates, the ONPG assay and the X-gal assay. ONPG or X-gal gives yellow or blue colour followed by enzymatic cleavage by β-gal. Our aim is to visually check whether the prepared nanoparticles are showing the activity of loaded β-gal or not. The nanoparticles free of unbound stabiliser after washing were tested for β-gal activity. 1 ml of 2 mM ONPG in water or 200 μL of 2% X-gal in DMF was added to 1 ml of the solution containing nanoparticles and the colour development after 1 h was visually confirmed.

To assess the substrate penetration in to the nanoparticles β-gal entrapped nanoparticles and β-gal enzyme were incubated in a microcentrifuge tube for 1 h and centrifuged at 30,000g for 5 min.

6.2.4. Delivery of β-gal loaded nanoparticles to rat

The procedure on rats was performed according to the United Kingdom Home Office Guide on the Operation of the Animals (Scientific Procedures) Act 1986. One batch of β-gal loaded nanoparticles (around 250 IU) suspended in 1 ml was administered to the male Sprague Dawley rat by oral gavage needle. After 2 h the rat was euthanized in a CO_2 chamber and the intestine was taken out. To another rat plain water was administered and euthanized in a CO_2 chamber after 2 h which served the purpose of negative control.

6.2.5. Histology

Rat intestine was taken out and small section was cut approximately 1 cm and washed properly with saline. Then the intestinal part was dropped in

2% X-gal in dimethyl formamide solution and incubated for 1 h. After 1 h the section was observed visually and cut through the longitudinal section into 2 pieces. One piece was dehydrated and embedded in wax using the process depicted in table 6.1.

Table 6.1: Wax embedding

Solution	Duration
100% ethanol	2 h
100% ethanol	2 h
50:50 ethanol:histoclear	1 h
100% histoclear	1 h
100% histoclear	1 h
Paraffin wax	2 h
Paraffin wax	2 h

6.2.5.1. Section cutting and slide preparation

Paraffin blocks were cut into 3 µm sections with a rotary microtome (RM 2125 RTF, Leica, Germany). Sections were floated on the surface of the water bath at around 50°C and mounted onto uncoated glass slides for staining. Slides were then heated in an oven at 60°C for 1 h to remove excess paraffin wax and stored at room temperature.

6.2.5.2. Nuclear fast red counter stain

Slides were counter stained for nuclei of the section with nuclear fast red. Nuclear fast red stain was prepared by dissolving 0.1 g of nuclear fast red and 5 g aluminium sulphate in 100 ml water and boiled. After cooling, the stain was added to the slides and washed with distilled water. Then the sections were mounted using DPX mountant and cover slips.

6.3. Results

The β-gal entrapped nanoparticles size after incubation with X-gal showed a second peak in the size distribution. The β-gal nanoparticles are 249 nm before [Fig 6.1A], and increased in size after incubation in X-gal

solution for 1 day, with a second peak for size distribution with an average size of 880 nm [Fig. 6.1B].

β-gal entrapped nanoparticles when incubated with X-gal gave blueish green colour and ONPG gave an yellow colour after 1 h [Fig. 6.2]. X-gal assay mixture of β-gal entrapped in nanoparticles was centrifuged at 30,000 g for 5 min. It can be observed in tube A of Fig. 6.3 that the formed precipitate from X-gal residing completely in the pellet as the supernatant appears clear without any colour. The same procedure when repeated with β-gal in solution added to assay mixture of X-gal does not result in clear supernatant.

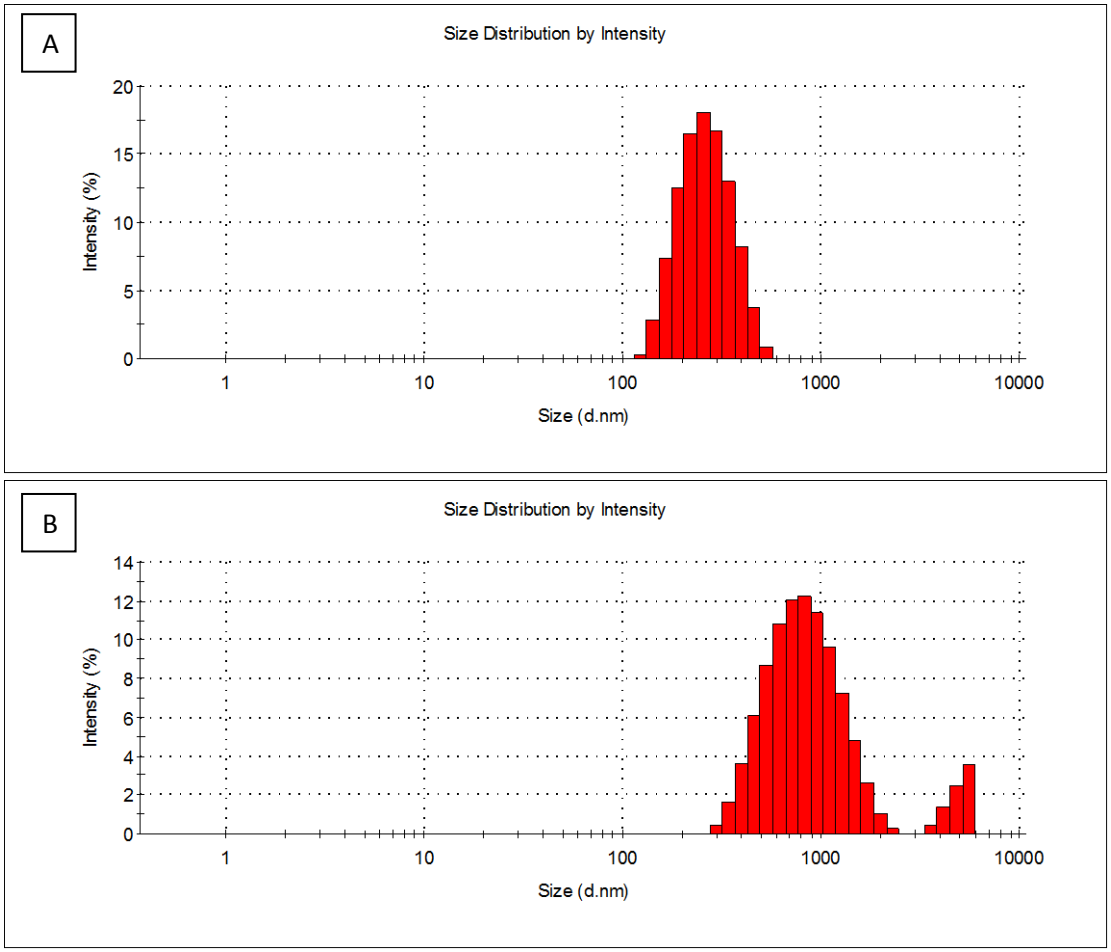


Figure 6.1. Particle size distribution of β-gal loaded nanoparticles (249 nm, PDI 0.058, zeta potential -0.590) (A), and β-gal loaded nanoparticles after incubation with X-gal for one day (880 nm, PDI 0.299, zeta potential 5.72) (B).

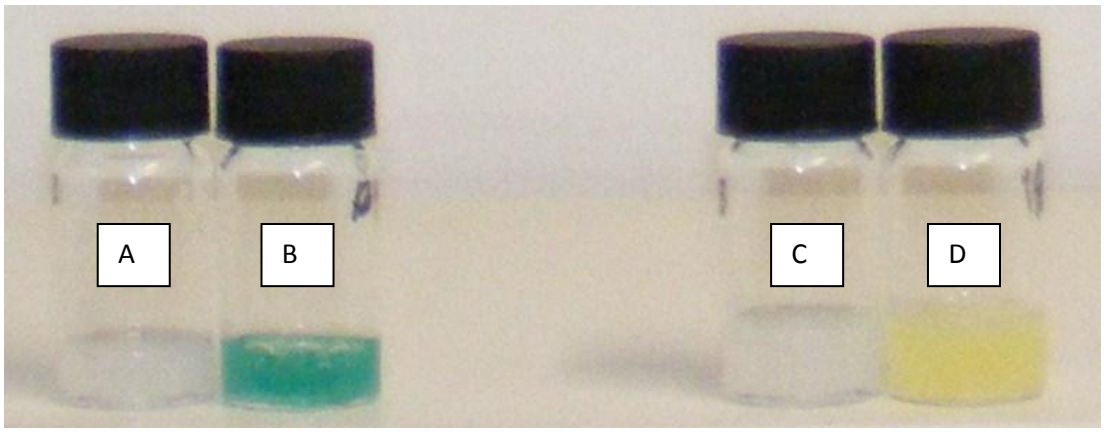


Fig. 6.2. Determination of β-gal enzyme activity in A) blank nanoparticles using X-gal B) β-gal loaded nanoparticles using X-gal C) blank nanoparticles using ONPG and D) β-gal loaded nanoparticles using ONPG. Nanoparticles loaded with β-gal are present in vials B&D showing the activity of the enzyme after 1 h incubation. This suggests that the nanoparticles not only retained the enzyme but they have protected its activity.

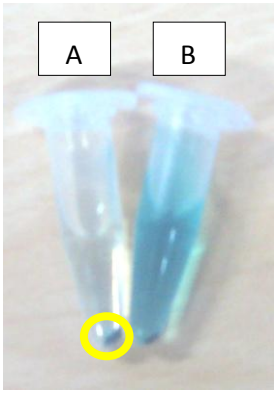


Fig. 6.3. X-gal assay mixture of β-gal nanoparticles (A) and β-gal solution (B) after centrifuging at 30,000 g for 5 min. It can be observed in tube A that the formed precipitate from X-gal residing completely in the pellet as the supernatant appears clear without any colour.

Fig. 6.4. The rat intestines showing β-gal loaded nanoparticles activity after 2 h of oral administration (left) and rat intestine from control without nanoparticles administration (right) when incubated with X-gal for 1 h.

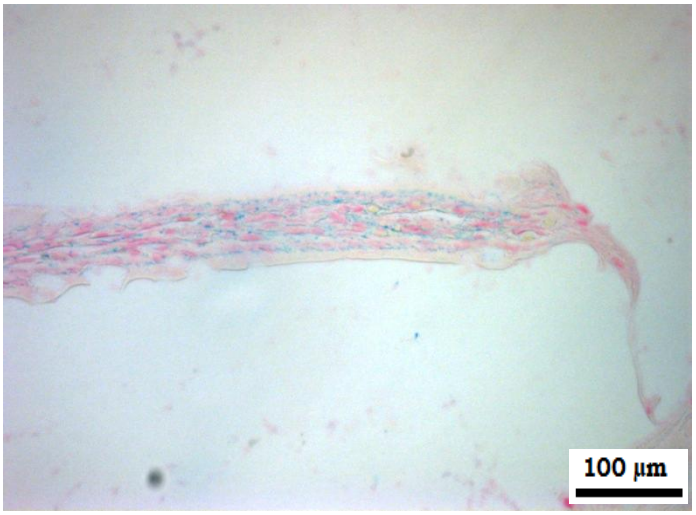


Fig.6.5. Nuclear fast red counter stained rat instestine showing the X-gal stain inside the villus. This figure suggests that β-gal nanoparticles are taken up intact by the villi of the intestine (40x2x10 magnification).

Rat intestine after incubation with X-gal for 2 h showed blue colour formation in the intestinal walls. The colour was inside the wall and after longitudinal sectioning of the intestine it was revealed that the colour was in the middle of the intestinal wall [Fig. 6.4]. The histological section of the intestine counter stained with nuclear fast red suggests that the staining was indeed inside the villi, which suggests the intact uptake of nanoparticles in to the intestinal wall [Fig. 6.5].

6.4. Discussion

6.4.1. X-gal staining of β-gal nanoparticles

X-gal gives an insoluble precipitate after enzymatic cleavage by β-gal. There are two possibilities of enzymatic action when the X-gal solution was incubated with nanoparticles loaded with β-gal. One is the enzyme has to be released from the nanoparticles or the substrate has to penetrate the nanoparticles matrix. The first possibility seems to be impossible as the polymer PLGA takes a long period to degrade and/or β-gal is a large molecule to come out from the nanoparticles without degradation. The other possibility left is the substrate penetration into the nanoparticles. The substrate penetration into the nanoparticles was confirmed by X-gal assay, where a β-gal solution and β-gal containing nanoparticles incubated with X-gal for 1 h. After that they were centrifuged at 30,000 g for 5 min in a centrifuge. The solution containing nanoparticles of β-gal resulted in a transparent solution with the nanoparticles pellet in dark blue colour, where as the solution containing β-gal resulted in blue coloured solution and pellet after centrifugation [Fig. 6.3]. From this it can be understood that the substrates can penetrate into the nanoparticles and can be cleaved by the enzyme entrapped in the nanoparticle matrix and remain in the nanoparticles. There is also a possibility of enzyme acting at the surface of

the nanoparticles. From the X-gal assay it can also be implied that the enzyme is retained in the polymer matrix and is not released. If the enzyme had been released from the particles the solution containing nanoparticles should have turned blue. Although this is not a confirmatory test, it gives a fair idea that enzyme is not released from the particles and further studies are needed to confirm that the enzyme is not released from the particles during the assay. Probably these β-gal nanoparticles can allow the detection of nanoparticles in the *in vivo* conditions for prolonged periods until the polymer degrades. In most of the drug delivery applications, in particular those used for oral drug delivery are expected to be in the circulation for prolonged duration and in some cases as high as 10-12 days. The circulation time and the sustainance of the release from the nanoparticles is found to be particle size dependent [Sahana *et al.* 2007; Italia *et al.* 2007]. *In vivo* or intracellular fate of the dyes and other agents used in different techniques to visualize the nanoparticles need to be established. The β-gal is an endogenous enzyme which has very few biocompatibility issues when compared to other dyes and agents. These particles can be used for the tissue distribution studies of the nanoparticles for prolonged periods of time. Most importantly the usage of β-gal assay which is very popular in molecular biology and genetics makes these particles highly useful. These particles can also be used for immobilization of β-gal and can be used as a therapeutic agent for the lactose intolerant patients.

6.4.2. Nanoparticles absorption and distribution

There has been a lot of support for the belief that nanoparticles deliver drugs in a sustained manner, when delivered orally, because of the direct uptake of nanoparticles into the lymphatic route and then sustains the release of the loaded active ingredients when delivered orally. The

absorption from the gut depends on the physical, chemical stability of the nanoparticles, transit time in the gut, transport through mucous etc [Florence 2005].

From the figures it is understandable that the β-gal loaded nanoparticles are penetrating into the intestinal mucosa of the rat. It is not just the mucoadhesion through which nanoparticles show their activity but they can be taken up directly into the gut wall. Other researchers also suggest the phenomenon of direct nanoparticles uptake through the gut associated lymphatic tissue (GALT) [Hussain *et al.* 2001]. If it is just the mucoadhesion, the X-gal stain would not be inside the gut wall [Fig. 6.4 & 6.5].

The tissue distribution studies described in the previous chapter using curcumin nanoparticles give insights into the nanoparticles distribution after oral administration. Nanoparticles were observed in the lung sections of rats which were given curcumin nanoparticles suggesting that systemic distribution of nanoparticles is possible with oral delivery [Fig. 5.6]. The analysis of curcumin in different tissues suggests that the nanoparticles extend the duration of resident time of the entrapped active ingredient in different organs in comparison to suspension formulation [Table 5.9]. Curcumin in its original form extensively distributed in the intestinal tissue, but with nanoparticles curcumin crossed the intestinal wall more efficiently which can be explained by the low distribution in the intestinal tissue and increased levels in the organs. Together β-gal nanoparticles and curcumin nanoparticles prove that the nanoparticles cross the intestinal wall intact efficiently and increase the distribution into systemic organs by increasing the absorption of the active ingredient entrapped in the nanoparticles.

6.5. Conclusion

β-gal loaded nanoparticles preserved the activity of enzyme which is evident from the colour development with ONPG and X-gal. The substrates penetrated the polymer matix PLGA and reach the enzyme to get cleaved. The particles get enlarged (up to 900 nm from 300 nm) with the substrate reaction which facilitates the visualization of the nanoparticles under the light microscope. From the experiments on the absorption through the intestines it can be proposed that the nanoparticles enter the intestinal walls intact. β-gal loaded nanoparticles can be useful in studying the absorption mechanism of the nanoparticles. From the results it can be concluded that the nanoparticles are taken up intact by the villi of the intestine.

7. GENERAL DISCUSSION

The main focus of the present research programme was on developing nanoparticulate delivery systems for EA, CoQ_{10} and curcumin which are otherwise difficult deliver through the oral route and attempts to study the absorption mechanisms of the nanoparticles using enzyme β-gal entrapped nanoparticles. Apart from using the well established EDE method for the preparation of nanoparticles (CoQ_{10} and curcumin nanoparticles) two different strategies were adapted in this research program which include co-encapsulation of antioxidants having different physicochemical properties (NanoCAPs of EA & CoQ_{10}) and the application of co-solvent in the preparation of nanoparticles using the EDE method (NanoCAPs of EA & CoQ_{10} and β-gal loaded nanoparticles). Both small molecules (EA, CoQ_{10} and curcumin) and a biomolecule (β-gal) were encapsulated successfully with considerable entrapment efficiency in to the nanoparticles of PLGA in the present research work.

The manufactured nanoparticle formulations were evaluated in three different disease models, i.e., NanoCAPs of EA & CoQ_{10} in high fat diet induced hyperlipidaemic rats, CoQ_{10} and curcumin nanoparticles in STZ induced diabetic rats and chronically hypoxic pulmonary hypertensive rats. NanoCAPs of EA & CoQ_{10} showed antihyperlipidaemic and endothelium protective effects in HFD fed rats. CoQ_{10} curcumin nanoparticles showed anti-inflammatory and anti-hyperlidaemic activities in STZ induced diabetic rats. However, curcumin nanoparticles seem to be more efficacious in terms of anti-inflammatory action than CoQ_{10} nanoparticles in STZ treated rats. CoQ_{10} and curcumin nanoparticles showed no beneficial effects in PH in terms of right ventricular hypertrophy and vascular remodelling.

Nanoparticle formulations of antioxidants are of particular interest as this delivery approach improves the therapeutic efficacy of the antioxidants which suffer from serious biopharmaceutical problems. The localisation of nanoparticles containing the antioxidants at the target site, i.e., the source of the oxidative stress is important to combat the oxidative stress related diseases. In our study CoQ_{10} and curcumin nanoparticles showed anti-hyperlipidaemic and anti-inflammatory activities in STZ induced diabetic rats, however, their effect was insignificant in amelioration of right ventricular hypertrophy and vascular remodelling in chronic hypoxic rats. The tissue distribution and histological examination in the lungs of chronic hypoxic rats suggest that curcumin was delivered to the lungs but might not have localised in the endothelium and other vascular layers which are the main sources of oxidative stress in PH, explaining the inability of these nanoparticles to normalise the vascular remodelling.

β-gal loaded nanoparticles showed interesting results in terms of their usage as a tool to study the absorption mechanism of the nanoparticles through oral route. β-gal loaded nanoparticles were shown to be taken up intact by the intestinal wall. These studies open up the possibility of using β-gal loaded nanoparticles to study the absorption, distribution in the cells and in animals.

8. GENERAL CONCLUSIONS & FUTURE DIRECTIONS

EDE method is a useful method to encapsulate either individual or combination of antioxidant molecules or an enzyme in lab scale. The use of co-solvent broadens the suitability of the EDE method for molecules with different physicochemical properties. The use of buffers as the continuous phase for the preparation of enzyme encapsulated nanoparticles can help in retaining the enzyme activity.

NanoCAPs of EA and CoQ_{10} were found to be effective in lowering the plasma lipid levels and increasing the endothelium dependent relaxation in the thoracic aorta of the high fat diet fed rats. NanoCAPs showed sustained action on cholesterol levels in comparison to the suspension formulation of the combination. CoQ_{10} and curcumin nanoparticles showed antihyperlipidemic activity in STZ induced diabetic rats. Curcumin nanoparticles showed better anti-inflammatory action by reducing IL-6, TNF-α and CRP whereas CoQ_{10} nanoparticles reduced only CRP in diabetic rats. CoQ_{10} and curcumin nanoparticles resulted in no action on the haematocrit, right ventricular hypertrophy and vascular remodelling developed in chronically hypoxic rats. Curcumin distribution studies in the chronically hypoxic rats suggest that the achieved lung levels (48±12 ng/g lung tissue) are not sufficient to counteract the effects of hypoxia. Curcumin tissue distribution analysis suggests altered tissue distribution of curcumin under hypoxia. Histological examination of curcumin nanoparticles (daily) treated chronic hypoxic rat lungs showed aggregates of curcumin nanoparticles suggesting that orally delivered nanoparticles reach the lungs. Experiments with β-gal nanoparticles suggested that nanoparticles can penetrate the gut wall intact carrying the encapsulated enzyme.

The studies discussed in chapters 2-6 can be extended for the better understanding of the role of nanoparticles in improving the efficacy of antioxidants in hyperlipidaemia, diabetes and pulmonary hypertension. The use of buffers in the continuous phase in preparing antioxidant encapsulated nanoparticles might help in increasing the stability of the antioxidants. Although the preparation of nanoparticles by EDE method is easy, harvesting the nanoparticles is a cumbersome process involving centrifugation and redispersion to separate the unentrapped active ingredient or stabiliser. Other ways of separation and concentrating nanoparticles need to be investigated.

CoQ_{10} is highly soluble in ethyl acetate which can facilitate higher initial loadings than that were used in the present research work. The initial loading of curcumin is limited to 15% w/v of polymer, as this is a high dose drug increasing the loading helps in lower number of batches to be prepared for a required dose. Or else scaling-up of the method has to be approached using different methods of homogenisation like the use of high pressure homogeniser. Methods other than emulsion diffusion evaporation can also be explored for the development of curcumin nanoparticles. The changes in the size, nanoparticles surface characteristics and release can be studied in simulated gastrointestinal fluids to understand *in vivo* performance.

Hyperlipidaemia studies indicated a potential activity of the combination of EA and CoQ_{10}. Even the CoQ_{10} nanoparticles and curcumin nanoparticles have been found to have hypolipidaemic actions in STZ induced diabetic rats. Studies using these nanoparticles can be extended to genetically modified animal models of hyperlipidaemia and atherosclerosis. Thorough understanding of the mechanisms of these nanoparticles in alleviating the hyperlipidaemia can also be studied.

The developed antioxidant nanoparticles can be tested in other models of oxidative stress induced disease states. Curcumin nanoparticles can also be used for the study of prevention of diabetes in STZ induced diabetic rat model as oxidative stress is implicated in diabetogenesis and intraperitoneal injection of curcumin showed benefits in prevention of diabetes in other studies [Kanitkar *et al.* 2008].

CoQ_{10} and curcumin nanoparticles showed no beneficial effects in PH. This could be due to the poor distribution of the antioxidants to the endothelium which is the main source of oxidative stress. Targeting approaches to the endothelium might be useful in demonstrating the activities of CoQ_{10} and curcumin in PH. There might be a variable kinetics of the antioxidants and nanoparticles in hypoxic conditions due to the altered blood flow to the major organs [Kuwahira *et al.* 1993]; thorough understanding of the distribution profiles of CoQ_{10} and curcumin using sensitive analytical methods is needed.

Nanoparticles might improve the performance of antioxidants by affecting one or more of the following properties like stability, absorption, metabolism, distribution or sustained release of the entrapped active moiety. To support the evidence of increased therapeutic efficacy, detailed stability studies and pharmacokinetics using sensitive analytical methods might be helpful.

The enzyme β-gal loaded nanoparticles showed positive results in the preliminary studies. These nanoparticles can be studied elaborately using different types of cell cultures to establish the uptake and cellular distribution or used for the *in vivo* distribution studies in major tissues.

REFERENCES

Abd, T. T., Jacobson, T. A., 2011. *Statin-induced myopathy: a review and update.* Expert opinion on drug safety, 10, 373-387.

Acosta, E., 2009. *Bioavailability of nanoparticles in nutrient & nutraceutical delivery.* Current Opinion in Colloid & Interface Science, 14, 3–15.

Adler, A. I., Stevens, R. J., Manley, S. E., Bilous, R. W., Cull, C. A., Homan, R. R., for the UKPDS Group, 2003. *Development & progression of nephropathy in type 2 diabetes, The United Kingdom Prospective Diabetes Study (UKPDS 64).* Kidney International, 63, 225–232.

Agbani, E. O., Coats, P., Mills, A., Wadsworth, R. M., 2011. *Peroxynitrite stimulates pulmonary artery endothelial and smooth muscle cell proliferation: Involvement of ERK and PKC.* Pulmonary pharmacology & therapeutics, 24, 100-109.

Ak, T., Gulcin, I., 2008. *Antioxidant & radical scavenging properties of curcumin.* Chemico-Biological Interactions, 174, 27–37.

Akiyama, T., Tachibana, I., Shirohara, H., Watanabe, N., Otsuki, M., 1996. *High-fat hypercaloric diet induces obesity, glucose intolerance and hyperlipidemia in normal adult male Wistar rat.* Diabetes Research and Clinical Practice, 31, 27-35.

Anand, I. S., 1994. *Hypoxia & the pulmonary circulation*. Thorax, 49, S19-S24.

Ankola, D. D., Viswanad, B., Bhardwaj, V., Ramarao, P., Kumar, M. N. V. R., 2007. *Development of potent oral nanoparticulate formulation of coenzyme Q10 for treatment of hypertension, Can the simple nutritional supplements be used as first line therapeutic agents for prophylaxis/therapy?* European Journal of Pharmaceutics & Biopharmaceutics, 67, 361-369.

Antons, K. A., Williams, C. D., Baker, S. K., & Phillips, P. S., 2006. *Clinical perspectives of statin-induced rhabdomyolysis*. The American Journal of Medicine , 119, 400–409.

Arun, K. H., Kaul, C. L., Ramarao, P., 2004. *High glucose concentration augments angiotensin II mediated contraction via AT1 receptors in rat thoracic aorta.* Pharmacological Research, 50, 561-568.

Atkinson, M. A., & Eisenbarth, G. S., 2001. *Type 1 diabetes, new perspectives on disease pathogenesis & treatment*. Lancet, 358, 221–229.

Austin, M. A., Hokanson, J. E., Edwards, K. L., 1998. *Hypertriglyceridemia as a cardiovascular risk factor.* American Journal of Cardiology, 81, 7B-12B.

Babu, P. S., Srinivasan, K., 1997. *Hypolipdemic action of curcumin, the active principle of turmeric (Curcuma longa) in streptozotocin induced diabetic rats.* Molecular and cellular biochemistry, 166, 169-175.

Badesch, D. B., Abman, S. H., Simonneau, G., Rubin, L. J., McLaughlin, V. V., 2007. *Medical therapy for pulmonary arterial hypertension, Updated ACCP evidence-based clinical practice guidelines*. Chest, 131, 1917–1928.

Bahl, O. P., Agrawal, K. M. L., 1969. *Glycosidases of Aspergillus niger*. Journal of Biological Chemistry 244, 2970.

Bala, I., Hariharan, S., Kumar, M. N. V. R., 2004. *PLGA nanoparticles in drug delivery, the state of the art*. Critical Reviews in Therapeutic Drug Carrier Systems, 21, 387-422.

Bala, I., Bharadwaj, V., Hariharan, S., Sitterberg, J., Bakowsky, U., Kumar, M. N. V. R., 2005. *Design of biodegradable nanoparticles, a novel approach to encapsulating poorly soluble phytochemical ellagic acid*. Nanotechnology, 16, 2819–2822.

Bala, I., Bhardwaj, V., Hariharan, S., Kumar, M. N. V. R., 2006a. *Analytical methods for assay of ellagic acid & its solubility studies*. Journal of Pharmaceutical and Biomedical Analysis, 40, 206–210.

Bala, I., Bhardwaj, V., Hariharan, S., Kharade, S. V., Roy, N., Kumar, M. N. V. R., 2006b. *Sustained release nanoparticulate formulation containing antioxidant-ellagic acid as potential prophylaxis system for oral administration*. Journal of Drug Targeting, 14, 27–34.

Bank, G., Kagan, D., Madhavi, D., 2011. *Coenzyme Q10: Clinical update and bioavailability.* Journal of Evidence-Based Complementary & Alternative Medicine, 16, 129-137.

Basu, A., Wilkinson, M., Penugonda, K., Simmons, B., Betts, N. M., Lyons, T. J., 2009. *Freeze-dried strawberry powder improves lipid profile and lipid peroxidation in women with metabolic syndrome: baseline and post intervention effects.* Nutrition Journal, 8, 43.

Baybutt, R. C., & Molteni, A., 1999. *Dietary β-carotene protects lung & liver parenchyma of rats treated with monocrotaline*. Toxicology, 137, 69-80.

Becker C, Hodenius M, Blendinger G, Sechi A, Hieronymus T, Muller-Schulte D, Schmitz-Rode T, Zenke M, 2007. *Uptake of magnetic nanoparticles into cells for cell tracking*. Journal of Magnetism and Magnetic Materials, 311, 234.

Belcaro, G., Cesarone, M. R., Dugall, M., Pellegrini, L., Ledda, A., Grossi, M. G., Togni, S., Appendino, G., 2010. *Efficacy and safety of Meriva®, a curcumin phosphatidyl complex, during extended administration in osteoarthritis patients.* Alternative Medicine Review, 15, 337-344.

Belle, T. L. V., Coppieters, K. T., & Von Herrath, M. G., 2011. *Type 1 Diabetes, Etiology, Immunology, & Therapeutic Strategies.* Physiological Reviews, 91, 79–118, 2011

Berbel-Garcia, A., Barbera-Farre, J., Etessam, J., Salio, A., Cabello, A., Gutierrez-Rivas, E., Campos, Y., 2004. *Coenzyme Q_{10} improves lactic acidosis, stroke-like episodes, & epilepsy in a patient with MELAS.* Clinical Neuropharmacology, 27, 187–191.

Bessler, H., Bergman, M., Blumberger, N., Djaldetti, M., Salman, H., 2009. *Coenzyme Q10 decreases TNF-alpha and IL-2 secretion by human peripheral blood mononuclear cells.* Journal of Nutritional Science and Vitaminology, (Tokyo). 56, 77-81.

Bhagavan, H. N., & Chopra, R. K., 2006. *Coenzyme Q10, Absorption, tissue uptake, metabolism & pharmacokinetics.* Free Radical Research, 40, 445-453.

Bhagavan, H. N., & Chopra, R. K., 2007. *Plasma coenzyme Q10 response to oral ingestion of coenzyme Q10 formulations*. Mitochondrion, 7, S78-S88.

Bhardwaj, V., Hariharan, S., Bala, I., Lamprecht, A., Kumar, N., Panchagnula, R., & Kumar, M. N. V. R., 2005. *Pharmaceutical aspects of polymeric nanoparticles for oral delivery*. Journal of Biomedical Nanotechnology, 1, 1–23.

Bhardwaj, V., Ankola, D. D., Gupta, S. C., Schneider, M., Lehr, C-M., Kumar, M. N. V. R., 2009. *PLGA nanoparticles stabilized with cationic surfactant: safety studies and application in oral delivery of paclitaxel to treat chemical-induced breast cancer in rat.* Pharmaceutical Research, 26, 2495–2503.

Bisht, S., Mizuma, M., Feldmann, G., Ottenhof, N. A., Hong, S. M., Pramanik, D., Chenna, V., Karikari, C., Sharma, R., Goggins, M. G., Rudek, M. A., Ravi, R., Maitra, A., Maitra, A., 2010. *Systemic administration of polymeric nanoparticle-encapsulated curcumin (NanoCurc) blocks tumor growth and metastases in preclinical models of pancreatic cancer.* Molecular Cancer Therapeutics. 8, 2255-2264.

Bisht, S., Feldmann, G., Soni, S., Ravi, R., Karikar, C., Maitra, A., Maitra, A., 2007. *Polymeric nanoparticle-encapsulated curcumin ("nanocurcumin"): a novel strategy for human cancer therapy.* Journal of Nanobiotechnology 2007, 5, 3.

Black, S. M., Fineman, J. R., 2006. *Oxidative & nitrosative stress in pediatric pulmonary hypertension, Roles of endothelin-1 & nitric oxide*. Vascular Pharmacology, 45, 308–316.

Bowers, R., Cool, C., Murphy, R. C., Tuder, R. M., Hopken, M. W., Flores, S. C. & Voelkel, N. F., 2004. *Oxidative stress in severe pulmonary hypertension*. American Journal of Respiratory and Critical Care Medicine, 169, 764-769.

Brennan, L. A., Steinhorn, R. H., Wedgwood, S., Mata-Greenwood, E., Roark, E. A., Russell, J. A., Black, S. M., 2003. *Increased superoxide generation is associated with pulmonary hypertension in fetal lambs, a role for NADPH Oxidase*. Circulation Research, 92, 683-691.

Brown, M. S., Goldstein, J. L., 1986. *A receptor-mediated pathway for cholesterol homeostasis.* Science, 232, 34-47.

Brufau, G., Canel, M. A., Rafecas, M., 2008. *Phytosterols: physiologic and metabolic aspects related to cholesterol-lowering properties.* Nutrition Research, 28, 217–225.

Bule, M. V., Singhal, R. S., Kennedy J. F., 2010. *Microencapsulation of ubiquinone-10 in carbohydrate matrices for improved stability.* Carbohydrate Polymers, 82, 1290–1296.

Cartiera, M. S., Johnson, K. M., Rajendran, V., Caplan, M. J., Saltzman, W. M., 2009. *The uptake and intracellular fate of PLGA nanoparticles in epithelial cells.* Biomaterials 30, 2790–2798.

Caso, G., Kelly, P., McNurlan, M. A., Lawson, W. E., 2007. *Effect of coenzyme q10 on myopathic symptoms in patients treated with statin medications*. American Journal of Cardiology, 9, 1497-1498.

Castelnau, P. A., Garrett, R. S., Palinski, W., Witztum, J. L., Campbell, I. L., & Powell, H. C., 1998. *Abnormal iron deposition associated with lipid peroxidation in transgenic mice expressing interleukin-6 in the brain*. Journal of Neuropathy and Experimental Neurology, 57, 268–282.

Chan, S. Y., Loscalzo, J., 2008. *Pathogenic mechanisms of pulmonary arterial hypertension*. Journal of Molecular and Cellular Cardiology, 44, 14–30.

Chen, H., Karne, R. J., Hall, G., *et al.* 2006. *High-dose oral vitamin C partially replenishes vitamin C levels in patients with type 2 diabetes and low vitamin C levels but does not improve endothelial dysfunction or insulin resistance.* Americal Journal of Physiology - Heart and Circulatory Physiology290, H137-H145.

Chin, K. M., Rubin, L. J., 2008. *Pulmonary arterial hypertension*. Journal of the American College of Cardiology, 51, 1527-1538.

Chisolm, G. M., Steinberg, D., 2001. *The oxidative modification hypothesis of atherogenesis: an overview.* Free Radical Biology and Medicine, 28, 1815–1826.

Chiu, S. S., Lui, E., Majeed, M., Vishwanatha, J. K., Ranjan, A. P., Maitra, A., Pramanik, D., Smith, J. A., Helson, L., 2011. Differential distribution of intravenous curcumin formulations in the rat brain. Anticancer Research, 3, 907-11.

Choi, B.S., Song, H. S., Kim, H. R., Park, T. W., Kim, T. D., Cho, B. J., Kim, C. J., Sim S. S., 2009. *Effect of coenzyme Q10 on cutaneous healing in skin-incised mice.* Arch. Pharmaceutical Research, 32, 907-913.

Cicero, A. F., Derosa, G., Miconi, A., Laghi, L., Nascetti, S., Gaddi, A., 2005, *Possible role of ubiquinone in the treatment of massive hypertriglyceridemia resistant to PUFA and fibrates.* Biomedicine and Pharmacotherapy, 59, 312-317.

Clinicaltrials.gov, Coenzyme Q-10 in the treatment of pulmonary arterial hypertension, http://clinicaltrials.gov/ct2/show/NCT01148836, accessed on 29-05-2011.

Colhoun, H. M., Schalkwijk, C., Rubens, M. B. & Stehouwer, C. D. A., 2002. *C-Reactive Protein in Type 1 Diabetes & Its Relationship to Coronary Artery Calcification*. Diabetes Care, 25, 1813–1817.

Cooper A, Nherera L, Calvert N, O'Flynn N, Turnbull N, Robson J, Camosso-Stefinovic J, Rule C, Browne N, Ritchie G, Stokes T, Mannan R, Brindle P, Gill P, Gujral R, Hogg M, Marshall T, Minhas R, Pavitt L, Reckless J, Rutherford A, Thorogood M, Wood D, 2008. *Clinical Guidelines and Evidence Review for Lipid Modification: cardiovascular risk assessment and the primary and secondary prevention of cardiovascular disease.* London: National Collaborating Centre for Primary Care and Royal College of General Practitioners.

Costacou, T., Ma, B., King, I. B., Mayer-Davis, E. J., 2008. Plasma and dietary vitamin E in relation to insulin secretion and sensitivity. Diabetes Obesity and Metabolism, 10, 223-228.

Crosswhite, P., Sun, Z., 2010. *Nitric oxide, oxidative stress and inflammation in pulmonary arterial hypertension.* Journal of Hypertension 28, 201–212.

Cummings, B. P., Stanhope, K. L., Graham, J. L., Evans, J. L., Baskin, D. G., Griffen, S. C., Havel, P. J., 2010. *Dietary fructose accelerates the development of diabetes in UCD-T2DM rats: amelioration by the antioxidant, α-lipoic acid.* American Journal of PhysiologyAmerican Journal of Physiology - Regulatory, Intergrative and Comparative Physiology, 298, R1343–R1350.

Davda, J., Labhasetwar ,V., 2002. *Chacterization of nanoparticle uptake by endothelial cells.* International Journal of Pharmaceutics 233, 51-59.

Davy G, Ciabattoni G, Consoli A, Mezzetti A, Falco A, Santarone S, *et al.* 1999. *In vivo formation of 8-iso-prostaglandin F2a and platelet activation in diabetes mellitus: effects of improved metabolic control and vitamin E supplementation.* Circulation , 99, 224–229.

DCCT Research Group, 1993. *The effect of intensive therapy of diabetes on the development & progression of long-term complications in insulin dependent diabetes mellitus*. New England Journal of Medicine, 329, 977.

Demiryurek, A. T., Karamsetty, M. R., McPhaden, A. R., Wadsworth, R. M., Kane, K. A., MacLean, M. R., 2000. *Accumulation of nitrotyrosine correlates with endothelial NO synthase in pulmonary resistance arteries during chronic hypoxia in the rat*. Pulmonary Pharmacology & Therapeutics, 13, 157–165.

Desai, M. P., Labhasetwar, V., Amidon, G. L., Levy, R. J., 1996. *Gastrointestinal uptake of biodegradable microparticles, effect of particle size*. Pharmaceutical Research, 13, 1838–1845.

Desai, M. P., Labhasetwar, V., Walter, E., Levy, R. J., & Amidon, G. L., 1997. *The mechanism of uptake of biodegradable microparticles in caco-2 cells is size dependent*. Pharmaceutical Research, 14, 1568-1573.

Devipriya, N., Sudheer A. R., Menon, V. P., 2007. *Dose-response effect of ellagic acid on circulatory antioxidants & lipids during alcohol-induced toxicity in experimental rats*. Fundamental & Clinical Pharmacology, 21, 621–630.

Doyle, B., Griffiths, L. A., 1980. *The metabolism of ellagic acid in the rat.* Xenobiotica, 10, 247-256.

Duarte, M. M. M. F., Moresco, R. N., Duarte, T., Santi, A., Bagatini, M. D., Da Cruz, I. B. M., Schetinger, M. R. C., Loro, V. L., 2010. *Oxidative stress in hypercholesterolemia and its association with Ala16Val superoxide dismutase gene polymorphism.* Clinical Biochemistry, 43, 1118–1123.

Dziubla T. D., Karim, A., Muzykantov, V. R., 2005. *Polymer nanocarriers protecting active enzyme cargo against proteolysis*. Journal of Controlled Release, 102, 427-439.

Dziubla, T. D., Shuvaev, V. V., Hong, N. K., Hawkins, B. J., Madesh, M., Takano, H., Simone, E., Nakada, M. T., Fisher, A., Albelda, S. M., Muzykantov, V. R., 2008. *Endothelial targeting of semi-permeable polymer nanocarriers for enzyme therapies.* Biomaterials, 29, 215–227.

Eddahibi, S., Adnot, S., 2006. *The serotonin pathway in pulmonary hypertension*. Archives des maladies du coeur et des vaisseaux (Abstract), 99, 621–625.

El-Azab, M. F., Attia, F. M., El-Mowafy, A. M., 2011. *Novel role of curcumin combined with bone marrow transplantation in reversing experimental diabetes, Effects on pancreatic islet regeneration, oxidative stress, & inflammatory cytokines*. European Journal of Pharmacology, 658, 41–48.

Elmedal, B., de Dam, M. Y., Mulvany, M. J., Simonsen, U., 2004. *The superoxide dismutase mimetic, tempol, blunts right ventricular hypertrophy in chronic hypoxic rats*. British Journal of Pharmacology, 141, 105–113.

Engler, M. M., Engler, M. B., Malloy, M. J., Chiu, E. Y., Schloetter, M. C., Paul, S. M., Stuehlinger, M., Lin, K. Y., Cooke, J. P., Morrow, J. D., Ridker, P. M., Rifai, N., Miller, E., Witztum, J. L., Mietus-Snyder, M., 2003. *Antioxidant vitamins C and E improve endothelial function in children with hyperlipidemia endothelial assessment of risk from lipids in youth (EARLY) trial.* Circulation, 108, 1059-1063.

Ernster, L., Dallner, G., 1995. *Biochemical, physiological & medical aspects of ubiquinone function*. Biochimica et Biophysica Acta, 1271, 195-204.

Figuero, E., Soory, M., Cerero, R., Bascones, A., 2006. *Oxidant/antioxidant interactions of nicotine, Coenzyme Q10, Pycnogenol & phytoestrogens in oral periosteal fibroblasts & MG63 osteoblasts.* Steroids, 71, 1062–1072.

Fike, C. D., Aschner, J. L., Slaughter, J. C., Kaplowitz, M. R., Zhang, Y., Pfister, S. L., 2011. *Pulmonary arterial responses to reactive oxygen*

species are altered in newborn piglets with chronic hypoxia-induced pulmonary hypertension. Pediatric Research, 70, 136-141.

Florence, A. T., 2005. *Nanoparticle uptake by the oral route: Fulfilling its potential?* Drug Discovery Today: Technologies, 2, 75-81.

Frankel, D. S., Wilson, P. W. F., Meigs, J. B., 2011. *Diabetes Mellitus and Cardiovascular Disease.* Atlas of atherosclerosis and metabolic syndrome, 227-252.

Frei, B., Kim, M., Ames, B. N., 1990. *Ubiquinol-10 is an effective lipid-soluble antioxidant at physiological concentrations*. Proceedings of the National Academy of Sciences USA, 87, 4879-4883.

Fresquet, F., Pourageaud, F., Leblais, V., Brandes, R. P., Savineau, J-P., Marthan, R., Muller, B., 2006. *Role of reactive oxygen species & gp91phox in endothelial dysfunction of pulmonary arteries induced by chronic hypoxia*. British Journal of Pharmacology, 148,714–723.

Festa, F., Aglitti, T., Duranti, G., Ricordy, R., Perticone, P., Cozzi, R., 2001. *Strong antioxidant activity of ellagic acid in mammalian cells in vitro revealed by the comet assay.* Anticancer Research, 21, 3903-3908.

Fumagalli, S., Fattirolli, F., Guarducci, L., Cellai, T., Baldasseroni, S., Tarantini, F., Bari, M. D., Masotti, G., Marchionni, N., 2011. *Coenzyme Q10 Terclatrate and Creatine in Chronic Heart Failure: A Randomized, Placebo-Controlled, Double-Blind Study.* Clinical cardiology, 34, 211-217.

Garcea, G., Jones, D. J. L., Singh, R., Dennison, A. R., Farmer, P. B., Sharma, R. A., Steward, W. P., Gescher, A. J., Berry, D. P., 2004. *Detection of curcumin and its metabolites in hepatic tissue and portal blood of*

patients following oral administration. British Journal of Cancer, 90, 1011 – 1015.

Garcea, G., Berry, D. P., Jones, D. J. L., Singh, R., Dennison, A. R., Farmer, P. B., Sharma, R. A., Steward, W. P., Gescher, A. J., 2005. *Consumption of the putative chemopreventive agent curcumin by cancer patients: Assessment of curcumin levels in the colorectum and their pharmacodynamic consequences.* Cancer Epidemiology, Biomarkers & Prevention, 14, 120-125.

Geraci, M. W., Gao, B., Shepherd, D. C., Moore, M. D., Westcott, J. Y., Fagan, K. A., Alger, L. A., Tuder, R. M., Voelkel, N. F., 1999. *Pulmonary prostacyclin synthase overexpression in transgenic mice protects against development of hypoxic pulmonary hypertension*. J. Clin. Invest., 103, 1509–1515.

Giaid, A., Yanagisawa, M., Langleben, D., Michel, R. P., Levy, R., Shennib, H., Kimura, S., Masaki, T., Duguid, W. P., Stewart, D. J., 1993. *Expression of endothelin-1 in the lungs of patients with pulmonary hypertension*. New England Journal of Medicine, 328, 1732–1739.

Girish, C., Pradhan, S. C., 2008. *Drug development for liver diseases: focus on picroliv, ellagic acid and curcumin.* Fundamental & Clinical Pharmacology, 22, 623–632.

Gökbel, H., Gergerlioğlu, H. S., Okudan, N., Gül, I., Büyükbaş, S., Belviranli, M., 2010. *Effects of coenzyme Q10 supplementation on plasma adiponectin, interleukin-6, and tumor necrosis factor-alpha levels in men.* Journal of Medicinal Food, 1:216-218.

Gopaul, N. K., Anggard, E. E., Mallet, A. I., Betteridge, D. J., Wolff, S. P., *et al.* 1995. *Plasma 8-epi-PGF2 alpha levels are elevated in individuals with NIDDM.* FEBS Letters, 368, 225–229.

Govender, T., Stolnik, S., Garnett, M. C., Illum, L., Davis, S. S., 1999. *PLGA nanoparticles prepared by nanoprecipitation, drug loading & release studies of a water soluble drug*. Journal Control Release, 57, 171-185.

Graham, D. J., Staffa, J. A., Shatin, D., 2004. *Incidence of hospitalized rhabdomyolysis in patients treated with lipid-lowering drugs.* The Journal of the American Medical Association, 292, 2585-2590.

Grama, C. N., Ankola, D. D., Kumar, M. N. V. R., 2011. *Poly(lactide-co-glycolide) nanoparticles for peroral delivery of bioactives.* Current Opinion in Colloid & Interface Science, 16, 238-245.

Grobe, A. C., Wells, S. M., Benavidez, E., Oishi, P., Azakie, A., Fineman, J. R., Black, S. M., 2006. *Increased oxidative stress in lambs with increased pulmonary blood flow & pulmonary hypertension, role of NADPH oxidase & endothelial NO synthase*. American Journal of Physiology, 290, L1069-L1077.

Gupta, S. K., Kumar, B., Nag, T. C., Agrawal, S. S., Agrawal, R., Agrawal, P., Saxena, R., & Srivastava, S., 2011. *Curcumin Prevents Experimental Diabetic Retinopathy in Rats Through Its Hypoglycemic, Antioxidant, & Anti-Inflammatory Mechanisms*. Journal of Ocular Pharmacology & Therapeutics, 27, 123-130.

Guzik, T. J., Mussa, S. M., Gastaldi, D., Sadowski, J., Ratnatunga, C., Pillai, R., Channon, K. M., 2002. *Mechanisms of increased vascular superoxide production in human diabetes mellitus:role of NAD(P)H oxidase and endothelial nitric oxide synthase.* Circulation, 105, 1656–1662.

Haan J. B., Jandeleit-Dahm, K. A., Allen, T. J., 2011. Role of oxidative stress and targeted antioxidant therapies in experimental models of diabetic complications. S. Basu and L. Wiklund (eds.), Studies on Experimental Models, Oxidative Stress in Applied Basic Research and Clinical Practice, Part 1, 3-38.

Halliwell, B., 2000. *The antioxidant paradox*. The Lancet, 355, 1179-1180.

Hariharan, S., Bhardwaj, V., Bala, I., Sitterberg, J., Bakowsky, U., Kumar, M. N. V. R., 2006. *Design of estradiol loaded PLGA nanoparticulate formulations:A potential oral delivery system for hormone therapy.* Pharmaceutical Research, 23, 184-195.

Hassoun, P., Thappa, V., Landman, M., Fanburg, B., 1992. *Endothelin 1 mitogenic activity on pulmonary artery smooth muscle cells & release from hypoxic endothelial cells*. Proceedings of the Society for Experimental Biology and Medicine, 199, 165–170.

Hayes, J. E., Allen, P., Brunton, N., O'Grady, M. N., Kerry, J. P., 2011. *Phenolic composition and in vitro antioxidant capacity of four commercial phytochemical products: Olive leaf extract (Olea europaea L.), lutein, sesamol and ellagic acid.* Food Chemistry, 126, 948–955.

Hayeshi, R., Mutingwende, I., Mavengere, W., Masiyanise, V., Mukanganyama, S., 2007. *The inhibition of human glutathione S-transferases activity by plant polyphenolic compounds ellagic acid & curcumin*. Food and Chemical Toxicology, 45, 286–295.

Hernell, O., Staggers, J. E., Carey, M.C., 1990. *Physical–chemical behavior of dietary & biliary lipids during intestinal digestion & absorption. 2. Phase analysis & aggregation states of luminal lipids during duodenal*

fat digestion in healthy adult human beings. Biochemistry, 29, 2041–2056.

Heyworth CM, Neumann EF, Wynn CH, 1981. *The stability & aggregation properties of human liver acid β-D-galactosidase*. Biochem. J., 193, 773.

Hirsch, I. B., 2009. *Clinical review, realistic expectations & practical use of continuous glucose monitoring for the endocrinologist*. The Journal of Clinical Endocrinology and Metabolism, 94, 2232–2238.

Hoshikawa, Y., Ono, S., Suzuki, S., Tanita, T., Chida, M., Song, C., Noda, M., Tabata, T., Voelkel, N.F., Fujimura, S., 2001. *Generation of oxidative stress contributes to the development of pulmonary hypertension induced by hypoxia*. Journal of Applied Physiology, 90, 1299–1306.

Hotamisligil, G. S. 1999. *Mechanisms of TNF-α-induced insulin resistance.* Exp. Clin. Endocrinol. Diabetes, 107, 119-125.

Hsia, J., MacFadyen, J. G., Monyak, J., Ridker, P. M., 2011. *Cardiovascular event reduction and adverse events among subjects attaining low-density lipoprotein cholesterol <50 mg/dl with rosuvastatin.* Journal of the American College of Cardiology, 57, 1666-1675.

Hsu, C. H., Cui, Z., Mumper, R. J., Jay, M., 2003. *Preparation & characterization of novel coenzyme Q10 nanoparticles engineered from microemulsion precursors*. AAPS PharmSciTech, 4, E32.

Hu, X., Guo, Y., Wang, L., Hua, D., Hong, Y., Li, J., 2011. *Coenzyme Q10 nanoparticles prepared by a supercritical fluid-based method*. The Journal of Supercritical Fluids, 57, 66-72.

Humbert, M., 2008. *Update in pulmonary arterial hypertension 2007.* American Journal of Respiratory and Critical Care Medicine, 177, 574–579.

Hung M-N, Xia Z, Hu N-T, Lee BH, 2001. *Molecular & Biochemical Analysis of Two β-Galactosidases from Bifidobacterium infantis HL96*. Applied and Environmental Microbiology, 67, 4256.

Hussain, N., Jaitley, V., Florence, A. T., 2001. *Recent advances in the understanding of uptake of microparticulates across the gastrointestinal lymphatics*. Advanced Drug Delivery Reviews, 50, 107–142.

Hwang, J. M., Cho, J. S., Kim, T. H., Lee, Y. I., 2010. *Ellagic acid protects hepatocytes from damage by inhibiting mitochondrial production of reactive oxygen species.* Biomedicine & Pharmacotherapy, 64, 264–270.

Ireson, C., Orr, S., Jones, D. J. L., Verschoyle, R., Lim, C-K., Luo, J-L., Howells, L., Plummer, S., Jukes, R., Williams, M., Steward, W. P., Gescher, A., 2001. *Characterization of metabolites of the chemopreventive agent curcumin in human and rat hepatocytes and in the rat in vivo, and evaluation of their ability to inhibit phorbol ester-induced prostaglandin E2 production.* Cancer Research, 61, 1058–1064.

Ireson, C. R., Jones, D. J. L., Orr, S., Coughtrie, M. W. H., Boocock, D. J., Williams, M. L., Farmer, P. B., Steward, W. P., Gescher, A. J., 2002. *Metabolism of the cancer chemopreventive agent curcumin in human & rat intestine*. Cancer Epidemiology Biomarkers & Prevention, 11, 105-111.

Ishii, H., Koya, D., & King, G.L., 1998. *Protein kinase C activation & its role in the development of vascular complications in diabetes mellitus*. Journal of Molecular Medicine, 76, 21-31.

Italia, J. L., Bhatt, D. K., Bhardwaj, V., Tikoo, K., Kumar, M. N. V. R., 2007. *PLGA nanoparticles for oral delivery of cyclosporine, Nephrotoxicity &*

pharmacokinetic studies in comparison to Sandimmune Neoral®, Journal of Controlled Release, 119, 197–206.

Jain, S. K., Rains, J., Croad, J., Larson, B., Jones, K., 2009. *Curcumin supplementation lowers TNF-α, IL-6, IL-8, and MCP-1 secretion in high glucose-treated cultured monocytes and blood levels of TNF-α, IL-6, MCP-1, glucose, and glycosylated hemoglobin in diabetic rats.* Antioxidants & Redox Signalling, 11, 241-249.

Jain, K. S., Kathiravan, M. K., Somani, R. S., Shishoo, J. C., 2007. *The Biology & chemistry of hyperlipidemia*. Bioorganic and Medicinal Chemistry, 15, 4674-4699.

Jang, E-M., Choi, M-S., Jung, U. J., Kim M-J., Kim, H-J., Jeon S-M., Shin, S-K., Seong C.N., Lee, M-K., 2008. *Beneficial effects of curcumin on hyperlipidemia & insulin resistance in high-fat–fed hamsters*, Metabolism Clinical & Experimental, 57, 1576–1583.

Jeremias, A., Soodini, G., Gelfand, E., Xu, Y., Stanton, R. C., Horton, E. S., Cohen, D. J., 2009. *Effects of N-acetyl-cysteine on endothelial function and inflammation in patients with type 2 diabetes mellitus*. Heart International, 4, e7, 25-29.

Johnson, S. R., Mehta, S., Granton, J. T., 2006. *Anticoagulation in pulmonary arterial hypertension, a qualitative systematic review*. European Respiratory Journal, 28, 999 –1004.

Jovanovic, S. V., Boone, C. W., Steenken, S., Trinoga, M., Kaskey, R. B., 2001. *How curcumin works preferentially with water soluble antioxidants.* Journal of the American Chemical Society, 123, 3064-3068.

Kannan, M. M., Quine, S. D., 2011. *Ellagic acid ameliorates isoproterenol induced oxidative stress: Evidence from electrocardiological, biochemical and*

histological study. European Journal of Pharmacology, Accepted manuscript.

Kang, J. H., 2003. *Modification and inactivation of human Cu,Zn-superoxide dismutase by methylglyoxal.* Molecules and Cells, 15:194; 2003.

Kanitkar, M., Gokhale, K., Galande, S., Bhonde, R. R., 2008. *Novel role of curcumin in the prevention of cytokine-induced islet death in vitro and diabetogenesis in vivo.* British Journal of Pharmacology, 155, 702–713.

Kansagara, D., Fu, R., Freeman, M., Wolf, F., Helfand, M., 2011. *Intensive Insulin Therapy in Hospitalized Patients: A Systematic Review.* Annals of Internal Medicine, 154, 268-282.

Kassab, E., McFarlane, S. I., & Sowers, J. R., 2001. *Vascular complications in diabetes & their prevention.* Vascular Medicine, 6,249-55.

Keys, A. 1980. Seven Countries, *A Multivariate Analysis of Death & Coronary Heart Disease.* Harvard University Press, Cambridge, MA.

Kishi, H., Kanamori, N., Nishii, S., Hiraoka, E., Okamoto, T., Kishi, T,. 1964. Metabolism of exogenous coenzyme Q10 in vivo and the bioavailability of coenzyme Q10 preparations in Japan. In: Folkers K, Yamamura Y, editors. Biomedical and clinical aspects of coenzyme Q. Amsterdam: Elsevier; p 131–142.

Koenig *et al.* 1999. *C-reactive protein, a sensitive marker of inflammation, predicts future risk of coronary heart disease in initially healthy middle-aged men, results from the MONICA (Monitoring Trends & Determinants in Cardiovascular Disease) Augsburg Cohort Study, 1984 to 1992*. Circulation, 99, 237–242.

Kommuru, T. R., Ashraf, M., Khan, M. A. & Reddy, I. K., 2001. *Self-emulsifying drug delivery systems (SEDDS) of coenzyme Q10,*

formulation development & bioavailability assessment. International Journal of Pharmaceutics, 212, 233-246.

Kontush, A., Reich, A., Baum, K., Spranger, T., Finckh, B., Kohlschutter, A., Beisiegel, U., 1997. *Plasma ubiquinol-10 is decreased in patients with hyperlipidaemia.* Atherosclerosis, 129, 119–126.

Kostapanos, M. S., Spyrou, A. T., Tellis, C. C., Gazi, I. F., Tselepis, A. D., Elisaf, M., Liberopoulos, E. N., 2011. *Ezetimibe treatment lowers indicators of oxidative stress in hypercholesterolemic subjects with high oxidative stress.* Lipids, 46, 341–348.

Kowluru, R. A., Kanwar, M., 2007. *Effects of curcumin on retinal oxidative stress and inflammation in diabetes.* Nutrition and Metabolism, 16, 4–8.

Kreuter, J., 1991. *Peroral administration of nanoparticles*. Advanced Drug Delivery Reviews, 7, 71–86.

Kucharska, J., Braunova, Z., Ulicna, O., Zlatos, L., & Gvozdjakova, A., 2000. *Deficit of coenzyme Q10 in heart & liver mitochondria of rats with streptozotocin-induced diabetes*. Physiological Research, 49, 411.

Kumari, A., Yadav, S. K., Yadav, S. C., 2010. *Biodegradable polymeric nanoparticles based drug delivery systems.* Colloids and Surfaces B: Biointerfaces, 75, 1–18.

Kunitomo, M., Yamaguchi, Y., Kagota, S., Otsubo, K., 2008. *Beneficial Effect of Coenzyme Q10 on Increased Oxidative and Nitrative Stress and Inflammation and Individual Metabolic Components Developing in a Rat Model of Metabolic Syndrome.* Journal of Pharmacological Sciences, 107, 128 – 137.

Kuwahira, I., Heisler, N., Piiper, J., Gonzalez, N. C., 1993. *Effect of chronic hypoxia on hemodynamics, organ blood flow and O2 supply in rats.* Respiration Physiology, 92, 227-238.

Kwon, S. S., Nam, Y. S., Lee, J. S., Ku, B. S., Han, S. H., Lee, J. Y., Chang, I. S., 2002. Preparation and characterization of coenzyme Q10-loaded PMMA nanoparticles by a new emulsification process based on microfluidization, Colloids and Surfaces A:Physicochemical and Engineering Aspects, 210, 95–104.

Lachmanova, V., Hnilickova, O., Povysilova, V., Hampl, V., Herget, J., 2005. *N-acetylcysteine inhibits hypoxic pulmonary hypertension most effectively in the initial phase of chronic hypoxia.* Life sciences, 77, 175–182.

Lai, S. K., Wang, Y. Y., Hanes, J., 2009. *Mucus-penetrating nanoparticles for drug and gene delivery to mucosal tissues.* Advanced Drug Delivery Reviews, 61, 158–171.

Lai, S. K., O'Hanlon, D. E., Harrold, S., Man, S. T., Wang, Y. Y., Cone, R., Hanes, J., 2007. *Rapid transport of large polymeric nanoparticles in fresh undiluted human mucus,* Proceedings of the National Acadamy of Sciences U. S. A. 104, 1482–1487.

Lai, Y. L., Wu, H. D., Chen, C. F., 1998. *Antioxidants attenuate chronic hypoxic pulmonary hypertension*. Journal of Cardiovascular Pharmacology, 32, 714–720.

Lampe, J. W., 1999. *Health effects of vegetables and fruit: assessing mechanisms of action in human experimental studies.* American Journal of Clinical Nutrition, 70, 475S-490S.

Landete, J. M., 2011. *Ellagitannins, ellagic acid and their derived metabolites: A review about source, metabolism, functions and health.* Food Research International, 44, 1150-1160.

Lamprecht, A., Ubrich, N., Perez, M. H., Lehr, C. M., Hoffman, M., Maincent, P., 2000. *Influences of process parameters on nanoparticle preparation performed by a double emulsion pressure homogenization technique*. International Journal of Pharmaceutics, 196, 177-182.

Lampson LA, Lampson MA, Dunne AD, 1993. *Exploiting the lacZ reporter gene for quantitative analysis of disseminated tumor growth within the brain, Use of the lacZ gene product as a tumor antigen, for evaluation of antigenic modulation, & to facilitate image analysis of tumor growth in situ*. Cancer Research, 53, 176.

Lardizabal, J. A., Deedwania, P. 2011. *Lipid-lowering therapy with statins for the primary and secondary prevention of cardiovascular disease.* Cardiology Clinics, 1, 87-103.

Larrosa, M., García-Conesa, M. T., Espín, J. C., Tomás-Barberán, F. A., 2010. *Ellagitannins, ellagic acid and vascular health.* Molecular Aspects of Medicine, 31, 513–539.

Lei, F., Zhang, X. N., Wang, W., Xing, D. M., Xie, W. D., Su, H., Du, L. J., 2007. *Evidence of anti-obesity effects of the pomegranate leaf extract in high-fat diet induced obese mice*. International Journal of Obesity, 31, 1023–1029.

Lei, F., Xing, D. M., Xiang, L., Zhao, Y-N., Wang, W., Zhang, L-J., Du, L-J., 2003. *Pharmacokinetic study of ellagic acid in rat after oral administration of pomegranate leaf extract.* Journal of

Chromatography B Analytical Technolology in the Biomedical and Life Sciences, 796, 189–194.

Li M-G., Lu, W-L., Wang, J-C., Zhang, X., Wang, X-Q., Zheng, A-P., Zhang, Q., 2007. *Distribution, transition, adhesion and release of insulin loaded nanoparticles in the gut of rats.* International Journal of Pharmaceutics, 329, 182–191.

Lin, Q., Wang, L. X., Chen, S. X., Zhou, X. F., Huang, X. Y., Fan, X. F., 2006. [Article in Chinese]. *Effect of curcumin on pulmonary hypertension and wall collagen of pulmonary arterioles of chronic hypoxic hypercapnic rats.* Zhongguo Ying Yong Sheng Li Xue Za Zhi., 3, 257-61. (Abstract)

Losso, J. N., Bansode, R. R., Trappey, A. II., Bawadi, H.A., Truax, R., 2004. *In vitro anti-proliferative activities of ellagic acid*. Journal of Nutritional Biochemistry, 15, 672–678.

Lü, J. M., Wang, X., Marin-Muller, C., Wang, H., Lin, P. H., Yao, Q., Chen, C., 2009. *Current advances in research and clinical applications of PLGA-based nanotechnology.* Expert Review of Molecular Diagnostics, 4, 325-41.

Lu, L., Zhang, Q., Pu, L. J., Xu, X. W., Zhang, R. Y., Zhang, J. S., Hu, J., Yang, Z. K., Lü, A. K., Ding, F. H., Shen, J., Chen, Q. J., Lou, S., Fang, D. H., Shen, W. F., 2007. *Elevation of tumor necrosis factor-α, interleukin-1β & interleukin-6 levels in aortic intima of Chinese Guizhou minipigs with streptozotocin-induced diabetes.* Chinese Medical Journal, 120, 479-484.

Madamanchi, N. R., Vendrov, A., Runge, M. S., 2005. *Oxidative Stress and Vascular Disease*. Arteriosclerosis Thrombosis and Vascular Biology, 25, 29-38.

Marinangeli, C. P. F., Jones, P. J. H., 2010. *Plant sterols, marine-derived omega-3 fatty acids and other functional ingredients: a new frontier for treating hyperlipidemia*. Nutrition & Metabolism, 7, 76-83.

Maroz, A., Anderson, R. F., Smith, R. A. J., Murphy, M. P., 2009. *Reactivity of ubiquinone & ubiquinol with superoxide & the hydroperoxyl radical, implications for in vivo antioxidant activity*. Free Radical Biology & Medicine, 46, 105–109.

Maruno, K., Absood, A., Said, S. I., 1995. *VIP inhibits basal & histamine stimulated proliferation of human airway smooth muscle cells*. American Journal of Physiology, 268, L1047–L1051.

Mason, N. A., Springall, D. R., Burke, M., Pollock, J., Mikhail, G., Yacoub, M. H., Polak, J. M., 1998. *High expression of endothelial nitric oxide synthase in plexiform lesions of pulmonary hypertension.* The Journal of Pathology, 185, 313–318.

Masri, F. A. Comhair, S. A. A., Dostanic-Larson, I., Kaneko, F. T., Dweik, R. A., Arroliga, A. C., Erzurum, S. C., 2008. *Deficiency of lung antioxidants in idiopathic pulmonary arterial hypertension.* Clinical and Translational Science, 1, 99-106.

Meena, A. K., Ratnam, D. V., Chandraiah, G., Ankola, D. D., Rao, P.R., Kumar, M. N. V. R., 2008. *Oral Nanoparticulate Atorvastatin Calcium is More Efficient & Safe in Comparison to Lipicure® in Treating Hyperlipidemia*. Lipids, 43, 231-241.

Meghana, K., Sanjeev, G., Ramesh, B., 2007. *Curcumin prevents streptozotocin-induced islet damage by scavenging free radicals: A prophylactic and protective role*. European Journal of Pharmacology, 577, 183–191.

Merrell, J. G., McLaughlin, S. W., Tie, L., Laurencin, C. T., Chen, A. F., Nair, L. S., 2009. *Curcumin-loaded poly(epsilon-caprolactone) nanofibres: diabetic wound dressing with anti-oxidant and anti-inflammatory properties.* Clinical and Experimental Pharmacology and Physiology, 12, 1149-1156.

Minko, T., Stefanov, A., Pozharov, V., 2002. *Selected Contribution, Lung hypoxia, antioxidant & antiapoptotic effects of liposomal α-tocopherol.* Journal of Applied Physiology, 93, 1550–1560.

Mittal, G., Sahana, D. K., Bhardwaj, V., Kumar, M. N. V. R, 2007. *Estradiol loaded PLGA nanoparticles for oral administration, Effect of polymer molecular weight & copolymer composition on release behavior in vitro & in vivo*. Journal of Controlled Release, 119, 77–85.

Mittal, G., Carswell, H., Brett, R., Currie, S., Kumar, M. N. V. R., 2011. *Development and evaluation of polymer nanoparticles for oral delivery of estradiol to rat brain in a model of Alzheimer's pathology.* Journal of Controlled Release, 150, 220–228.

Modi, K. P., Vishwakarma, S. L., Goyal, R. K., Bhatt, P. A., 2007. *Effects of coenzyme Q10 on lipid levels and antioxidant defenses in rats with fructose induced hyperlipidemia and hyperinsulinaemia.* The Internet Journal of Pharmacology. 2007, 5.

Moller, D. E., 2000. *Potential role of TNF-α in the pathogenesis of insulin resistance & type 2 diabetes*. Trends in Endocrinology and Metabolism, 11, 212-217.

Molteni, A., Herndon, B. L., Kamal, A., Castellani, W. J., Reppert, S., Xue, Y., Humbehr, J., Baybutt, R. C., 2004. *Effect of the antioxidant α-tocopherol in an experimental model of pulmonary hypertension &*

fibrosis, administration of monocrotaline. Nutrition Research, 24, 707–720.

Moriel, P., Plavnik, F. L., Zanella, M. T., Bertolami, M. C., Abdalla, D. S. P., 2000. *Lipid peroxidation and antioxidants in hyperlipidemia and hypertension.* Biological Research, 33, 105-112.

Munjal, B., Pawar, Y. B., Patel, S. B., Bansal, A. K., 2011. *Comparative oral bioavailability advantage from curcumin formulations.* Drug Delivery and Translational Research, DOI 10.1007/s13346-011-0033-3.

Murakami, H., Kawashima, Y., Niwa, T., Hino, T., Akeuchi, H. T., Obayashi, M. K., 1997. *Influence of the degrees of hydrolyzation & polymerization of poly(vinylalcohol) on the preparation & properties of poly(lactide-co-glycolide) nanoparticle.* International Journal of Pharmaceutics, 149, 43-49.

Murugan, V., Mukherjee, K., Maiti, K., Mukherjee, P. K., 2009. *Enhanced oral bioavailability and antioxidant profile of ellagic acid by phospholipids.* Journal of Agricultural and Food Chemistry, 57, 4559-4565.

Muthenna, P., Suryanarayana, P., Gunda, S. K., Petrash, J. M., Reddy, G. B., 2009. *Inhibition of aldose reductase by dietary antioxidant curcumin: Mechanism of inhibition, specificity and significance.* FEBS Letters, 583, 3637–3642.

Nakanishi, K., Tajima, F., Nakamura, A., Yagura, S., Ookawara, T., Yamashita, H., Suzuki, K., Taniguchi, N., Ohno, H., 1995. *Effects of hypobaric hypoxia on antioxidant enzymes in rats.* Journal of Physiology, 489, 869-876.

Nicholls, M. G., Lainchbury, J. G., Lewis, L. K., Mcgregor, D. O., Richards, A. M., Troughton, R. W., Yandle, T. G., 2001. *Bioactivity of*

adrenomedullin & proadrenomedullin N-terminal 20 peptide in man. Peptides, 22, 1745–1752.

Nigris, F., Williams-Ignarro, S., Lerman, L. O., Crimi, E., Botti, C., Mansueto, G., D'Armiento, F. P., De Rosa, G., Sica, V., Ignarro, L. J., Napoli, C., 2005. *Beneficial effects of pomegranate juice on oxidation-sensitive genes and endothelial nitric oxide synthase activity at sites of perturbed shear stress.* Proceedings of the American Academy of SciencesUSA., 102, 4896-4901.

Nishikawa, T., Edelstein, D., Du, X. L., Yamagishi, S., Matsumura, T., Kaneda, Y., Yorek, M. A., Beebe, D., Oates, P. J., Hammes, H. P., Giardino, I., Brownlee, M., 2000. *Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage.* Nature, 404, 787–790.

Nishikimi, T., Nagata, S., Sasaki, T., Tomimoto, S., Matsuoka, H., Takishita, S., Kitamura, K., Miyata, A., Matsuo, H., Kangawa, K., 1997. *Plasma concentrations of adrenomedullin correlate with the extent of pulmonary hypertension in patients with mitral stenosis*. Heart, 78, 390–395.

O'Brien, T., Nguyen, T. T., & Zimmerman, B. R., 1998. *Hyperlipidemia & diabetes mellitus.* Mayo Clinic Proceedings, 73(10), 969-976.

Ogawa, Y., Kanatsu, K., Iino, T., Kato, S., Jeong, Y. I., Shibata, N., Takada, K., Takeuchi, K., 2002. *Protection against dextran sulfate sodium-induced colitis by microspheres of ellagic acid in rats.* Life Sciences, 71, 827-839.

Ohashi, H., Takami, T., Koyama, N., Kogure, Y., Ida, K., 1984. *Aqueous solution containing ubidecarenone*. US4483873.

Okamoto, T., Matsuya, T., Fukunaga, Y., Kishi, T., Yamagami, T., 1989. *Human serum ubiquinol-10 levels & relationship to serum lipids.* International Journal for Vitamin and Nutritional Research, 59,288-292.

Ozawa, Y., Mizushima, Y., Koyama, I., Akimoto, M., Yamagata, Y., Hayashi, H., Murayama, H., 1986. *Intestinal absorption enhancement of coenzyme Q10 with a lipid microsphere.* Arzneimittelforschung, 36,689-690.

Pan, M-H., Huang, T-M., Lin, J-K., 1999. *Biotransformation of curcumin through reduction & glucuronidation in mice.* Drug Metabolism and Disposition, 27, 486-494.

Panagi Z, Beletsi A, Evangelatos G, Livaniou E, Ithakissios DS, Avgoustakis K, 2001. *Effect of dose on the biodistribution & pharmacokinetics of PLGA & PLGA–mPEG nanoparticles*. International Journal of Pharmaceutics 221, 143.

Panchal, H. D., Vranizan, K., Lee, C. Y., Ho, J., Ngai, J., Timiras, P. S., 2008. *Early anti-oxidative & anti-proliferative curcumin effects on neuroglioma cells suggest therapeutic targets.* Neurochemical Research, 33, 1701-1710.

Panyam J, Sahoo SK, Prabha S, Bargar T, Labhasetwar V, 2003. *Fluorescence & electron microscopy probes for cellular & tissue uptake of poly(d,l-lactide-co-glycolide) nanoparticles*. International Journal of Pharmaceutics, 262.

Patterson, C. C., Dahlquist, G. G., Gyurus, E., Green, A., Soltesz, G., (the EURODIAB study Group), 2009. *Incidence trends for childhood type 1 diabetes in Europe during 1989–2003 & predicted new cases 2005–20, a multicentre prospective registration study*. Lancet, 373, 2027–2033.

Peek L. J., Middaugh C. R., Berkland C., 2008. *Nanotechnology in vaccine delivery*. Advanced Drug Delivery Research, 60, 915.

Plapied, L., Duhem, N., des Rieux, A., Préat, V., 2011. *Fate of polymeric nanocarriers for oral drug delivery.* Current Opinion in Colloid & Interface Science, 16, 228–237.

Prabha, S., Zhou, W-Z., Panyam, J., Labhasetwar, V., 2002. *Size-dependency of nanoparticle-mediated gene transfection: studies with fractionated nanoparticles.* International Journal of Pharmaceutics, 244, 105–115.

Press, R.E., Hardcastle, D., 1969. *Some physic-chemical properties of ellagic acid.* Journal of Applied Chemistry, 19, 247-251.

Preston, I. R., 2008. *Combination therapies in pulmonary arterial hypertension*. Advances in Pulmonary Hypertension, 7, 235-240.

Quiles, J. L., Ochoa, J. J., Huertas, J. R., Mataix, J., 2004. *Coenzyme Q supplementation protects from age-related DNA double-strand breaks & increases lifespan in rats fed on a PUFA-rich diet*. Experimental Gerontology, 39,189–194.

Ragheb, A., Attia, A., Elbarbry, F., Prasad, K., Shoker, A., 2011. *Attenuated combined action of cyclosporine A and hyperlipidemia on atherogenesis in rabbits by thymoquinone.* Evidence-Based Complementary and Alternative Medicine, article in press.

Rahimi, R., Nikfar, S., Larijani, B., Abdollahi, M., 2005. *A review on the role of antioxidants in the management of diabetes and its complications.* Biomedicine & Pharmacotherapy, 59, 365–373.

Rains, J. L., Jain, S. K., 2011. *Oxidative stress, insulin signaling, and diabetes.* Free Radical Biology & Medicine, 50, 567–575.

Rajadurai, M., Prince, S. M. P., 2006. *Preventive effect of naringin on lipid peroxides and antioxidants in isoproterenol-induced cardio toxicity in Wistar rats: biochemical and histopathological evidences.* Toxicology 228, 259–268.

Rao, J. P., Geckeler, K. E., 2011. *Polymer nanoparticles: Preparation techniques and size-control parameters.* Progress in Polymer Science, 36, 887–913.

Rathore, N., John, S., Kale, M., Bhatnagar, D., 1998. *Lipid peroxidation and antioxidant enzymes in isoproterenol induced oxidative stress in rat tissues.* Pharmacological Research. 38, 297–303.

Ratnam, D. V., Chandraiah, G.,Sonaje, K., Viswanad, B., Bhardwaj, V., Ramarao, P.,Kumar, M. N. V. R., 2008. *A potential therapeutic strategy for diabetes and its complications in the form of co-encapsulated antioxidant nanoparticles (NanoCAPs) of ellagic acid and Coenzyme Q_{10}: preparation and evaluation in streptozotocin induced diabetic rats.* Journal of Biomedical Nanotechnology, 4, 33–43.

Ratnam, D. V., Ankola, D. D., Bhardwaj, V., Sahana, D. K., Kumar, M. N. V. R., 2006a. *The role of antioxidants in prophylaxis & therapy, a pharmaceutical perspective*, Journal of Controlled Release, 113, 189-207.

Reddy, A. C., Lokesh, B. R., 1994. *Effect of dietary turmeric (Curcuma longa) on iron induced lipid peroxidation in the rat liver*. Food and Chemical Toxicology 32, 279–283.

Reddy, A. C., Lokesh, B. R., 1992. *Studies on spice principles as antioxidants in the inhibition of lipid peroxidation of rat liver microsomes*. Molecular and Cellular Biochemistry, 111, 117–124.

Riccioni, G., Bucciarelli, T., Mancini, B., Di Ilio, C., Capra, V., D'Orazio, N., 2007. *The role of the antioxidant vitamin supplementation in the prevention of cardiovascular diseases.* Expert Opinion on Investigational Drugs, 16, 25-32.

Rice-Evans, C. A., Miller, N. J., Paganga, G., 1996. *Structure–antioxidant activity relationships of flavonoids and phenolic acids.* Free Radical Biology and Medicine, 20, 933–956.

Rolland A, 1988. *Pharmacokinetics & tissue distribution of doxorubicin-loaded polymethacrylic nanoparticles in rabbits*. International Journal of Pharmaceutics, 42, 145-154..

Romanos MA, Scorer CA, Clare JJ, 1992. *Foreign gene expression in yeast, a review*. Yeast 8, 423-488.

Ruan, C-H., Dixon, R. A. F., Willerson, J. T., Ruan, K-H., 2010. *Prostacyclin Therapy for Pulmonary arterial hypertension.* Texas Heart Institute Journal, 37, 391-399.

Rundek, T., Naini, A., Sacco, R., Coates, K., DiMauro, S., 2004. *Atorvastatin decreases coenzyme Q10 level in blood of patients at risk for cardiovascular disease and stroke.* Archives of Neurology, 61, 889-892.

Sahana D, Mittal G, Bhardwaj V, Kumar MNVR, 2008. *PLGA Nanoparticles for oral administration of hydrophobic drugs, influence of organic solvent on nanoparticle formation & release behavior in vitro & in vivo using estradiol as a model drug*. Journal of Pharmaceutical Sciences, 97, 1530-1542.

Sahoo, S. K., Panyam, J., Prabha, S., Labhasetwar, V., 2002. *Residual polyvinyl alcohol associated with poly (DL-lactide-co-glycolide) nanoparticles affects their physical & cellular uptake properties.* Journal of Controlled Release, 82, 105-114.

Sanders, K. A., Hoidal, J. R., 2007. *The NOX on pulmonary hypertension.* Circulation Research, 101, 224–226.

Sandur, S. K., Pandey, M. K., Sung, B., Ahn, K. S., Marakami, A., Sethi, G., Limtrakul, P., Badbaev, V., Aggarwal, B. B., 2007. *Curcumin demethoxycurcumin, bisdemethoxycurcumin, tetrahydrocurcumin and turmerones differentially regulate anti-inflammatory and antiproliferative responses through a ROS independent mechanism.* Carcinogensis, 43, 568–580.

Saraf, A. S., 2010. *Applications of novel drug delivery system for herbal formulations*. Fitoterapia, 81, 680–689.

Schubert S. Y., Neeman, I., Resnick N., 2002. *A novel mechanism for the inhibition of NF-κB activation in vascular endothelial cells by natural antioxidants*. The FASEB Journal, 16,1931-1933.

Sekhar, R. V., Mckay, S. V., Patel, S. G., Guthikonda, A. P., Reddy, V. T., Balasubramanyam, A., Jahoor, F., 2011. *Glutathione synthesis is diminished in patients with uncontrolled diabetes and restored by dietary supplementation with cysteine and glycine.* Diabetes Care, 34, 162–167.

Selivanov, V. A., Votyakova, T. V., Pivtoraiko, V. N., Zeak, J., Sukhomlin, T., Trucco, M., Roca, J., Cascante, M., 2011. *Reactive oxygen species production by forward and reverse electron fluxes in the mitochondrial respiratory chain.* PLoS Computational Biology, 7, 1-17.

Sena, C. M., Nunes, E., Gomes, A., Santos, M. S., Proença, T., Martins, M. I., Seiça, R. M., 2008. *Supplementation of coenzyme Q10 and α-tocopherol lowers glycated hemoglobin level and lipid peroxidation in pancreas of diabetic rats.* Nutrition Research, 28, 113–121.

Sengupta, S., Sasisekharan, R., 2007. *Exploiting nanotechnology to target cancer*. British Journal of Cancer, 96, 1315.

Setthacheewakul, S., Mahattanadul, S., Padoongsombat, N., Pichayakorn, W., Wiwattanapatapee, R., 2010. *Development and evaluation of self-microemulsifying liquid and pellet formulations of curcumin, and absorption studies in rats.* European Journal of Pharmaceutics and Biopharmaceutics, 76, 475–485.

Shaikh, J., Ankola, D. D., Beniwal, V., Singh, D. and Kumar, M. N. V. R., 2009. *Nanoparticle encapsulation improves oral bioavailability of curcumin by at least 9 fold when compared to curcumin administered with piperine as absorption enhancer.* European Journal of Pharmaceutical Sciences, 37, 223-230.

Sharma, R. A., Euden, S. A., Platton, S. L., Cooke, D. N., Shafayat, A., Hewitt, H. R., Marczylo, T. H., Morgan, B., Hemingway, D., Plummer, S. M., Pirmohamed, M., Gescher, A. J., Steward, W. P., 2004. *Phase I clinical trial of oral curcumin: Biomarkers of systemic activity and compliance.* Clinical Cancer Research, 10, 6847–6854.

Sharma, R. A., Steward, W. P., Gescher, A. J., 2007. *Pharmacokinetics & pharmacodynamics of curcumin.* Advances in Experimental Medicine and Biology, 595, 453-470.

Sheikh-Ali, M., Chehade, J. M., Mooradian, A. D., 2009. *The Antioxidant Paradox in Diabetes Mellitus.* American Journal of Therapeutics, 18, 266-278.

Shen, L., Ji, H.-F., 2007. *Theoretical study on physicochemical properties of curcumin.* Spectrochimica Acta Part A, Molecular & Biomolecular Spectroscopy, 67, 619-623.

Shin, S-B., Cho, H-Y., Kim, D-D., Choi, H-G., Lee, Y-B., 2010. *Preparation and evaluation of tacrolimus-loaded nanoparticles for lymphatic delivery.* European Journal of Pharmaceutics and Biopharmaceutics, 74, 164–171.

Singh, R. B., Shinde, S. N., Chopra, R. K., Niaz, M. A., Thakur, A. S., Onouchi, Z., 2000. *Effect of coenzyme Q_{10} on experimental atherosclerosis and chemical composition and quality of atheroma in rabbits.* Atherosclerosis, 148, 275–282.

Sirtori, C. R., 1993. *Tissue slectivity of hydroxylglutaryl coenzyme A(HMG CoA) reductase inhibitors*. Pharmacology & Therapeutics, 60, 431-459.

Soderman, C., Eriksson, L. S., Juhlin-Dannfelt, A., Lundberg, J. M., Broman, L., Holmgren, A., 1993. *Effect of vasoactive intestinal polypeptide (VIP) on pulmonary ventilation-perfusion relationships & central haemodynamics in healthy subjects.* Clinical Physiology, 13, 677–685.

Sohet, F. M., Neyrinck, A. M., Pachikian, B. D., Backer, F. C., Bindels, L. B., Niklowitz, P., Menke, T., Cani, P. D., Delzenne, N. M., 2009. *Coenzyme Q10 supplementation lowers hepatic oxidative stress and inflammation associated with diet-induced obesity in mice.* Biochemical Pharmacology, 78, 1391–1400.

Sonaje, K., Italia, J., Sharma, G., Bhardwaj, V., Tikoo, K. & Kumar, M. N. V. R., 2007. *Development of biodegradable nanoparticles for oral delivery of ellagic acid & evaluation of their antioxidant efficacy against cyclosporine A-induced nephrotoxicity in rats*. Pharmaceutical Research, 24, 899-908.

Sood, B. G., Wykes, S., Landa, M., Jesus, L. D., Rabah, R., 2011. *Expression of eNOS in the lungs of neonates with pulmonary hypertension.* Experimental and Molecular Pathology, 90, 9–12.

Sorescu, D., Griendling, K. K., 2002. *Reactive oxygen species, mitochondria and NADPH oxidases in the development and progression to heart failure.* Congestive Heart Failure, 8, 132-140.

Souich, P., Clozel, J.P., Saunier, C., Hartemann, D., Schrijen, F., Amend, P., 1985. The influence of hypoxemia on tritiated digoxin plasma kinetics and tissue distribution in the conscious dog. American Review of Respiratory Disease, 132, 504-509.

Souich, P., Sauniert, C., Hartemann, D., Allam, M., 1992. *Effect of acute and chronic moderate hypoxia on the kinetics of lidocaine and its metabolites and on regional blood flow*. Pulmonary Pharmacology, 5, 9-16.

Srinivasan, K., Viswanad, B., Asrat, L., Kaul, C. L., Ramarao, P., 2005. *Combination of high-fat diet-fed and low-dose streptozotocin-treated rat: a model for type 2 diabetes and pharmacological screening.* Pharmacological Research, 52, 313-320.

Srivastava, S., Chandrasekar, B., Gu, Y., Luo, J., Hamid, T., Hill, B.G., Prabhu, S.D., 2007. *Downregulation of CuZn-superoxide dismutase contributes to beta-adrenergic receptor-mediated oxidative stress in the heart.* Cardiovascular Research, 74, 445–455.

Steinberg, D., 2006a. *An interpretive history of the cholesterol controversy, part IV: The 1984 Coronary Primary Prevention Trial ends it—almost.* The Journal of Lipid Research, 47, 1–14.

Steinberg, D., 2006b. *An interpretive history of the cholesterol controversy, part V: The discovery of the statins and the end of the controversy.* The Journal of Lipid Research, 47, 1339–1351.

Steinberg, D., & Witztum, J. L., 2002. *Is the oxidative modification hypothesis relevant to human atherosclerosis? Do the antioxidant trials conducted to date refute the hypothesis?* Circulation, 105, 2107–2111.

Stivaktakis N, Nikou K, Panagi Z, Beletsi A, Leontiadis L, Avgoustakis K, 2004. *PLA & PLGA microspheres of β-galactosidase, effect of formulation factors on protein antigenicity & immunogenicity.* Journal of Biomedical Materials Research, 70A, 139-148.

Sudheer, A. R., Muthukumaran, S., Devipriya, N., Menon, V. B., 2007. *Ellagic acid, a natural polyphenol protects rat peripheral blood lymphocytes against nicotine-induced cellular and DNA damage in vitro: With the comparison of N-acetylcysteine.* Toxicology, 230, 11–21.

Sun W, Wang H, Xie C, Hu Y, Yang X, Xu H, 2006. *An attempt to directly trace polymeric nanoparticles in vivo with electron microscopy.* Journal of Controlled Release, 115, 259-265.

Tcnnesen, H. H., Greenhill, J. V., 1992. *Studies on curcumin & curcuminoids. XXII, Curcumin as a reducing agent & as a radical scavenger.* International Journal of Pharmaceutics, 87, 79-87.

Thompson, G. R., 2004. *Management of dyslipidaemia.* Heart, 90, 949-55.

Thompson, P. D., Clarkson, P., Karas, R. H., 2003. *Statin-associated myopathy.* JAMA, 289, 1681-1690.

Tiano, L., Belardinelli, R., Carnevali, P., Principi, F., Seddaiu, G., Littarru, G. P., 2007. *Effect of coenzyme Q10 administration on endothelial function and extracellular superoxide dismutase in patients with ischaemic heart disease: a double-blind, randomized controlled study.* European Heart Journal, 28, 2249–2255.

Tobio, M., Sanchez, A., Vila, A., Soriano, I., Evora, C., Vila-Jato, J. L., Alonso, M. J., 2000. *The role of PEG on the stability in digestive fluids and in*

vivo fate of PEG-PLA nanoparticles following oral administration. Colloids and Surfaces B: Biointerfaces 18, 315-323.

Tomono, Y., Hasegawa, J., Seki, T., Motegi, K., Morishita, N., 1986. International Journal of Clinical Pharmacology, Therapy and Toxicology, 24, 536-541.

Touyz, R. M., Briones, A. M., Sedeek, M., Burger, D., Montezano, A. C., 2011. *NOX isoforms and reactive oxygen species in vascular health.* Molecular Interventions, 11, 27-35.

Tsuneki, H., Sekizaki, N., Suzuki, T., Kobayashi, S., Wada, T., Okamoto, T., Kimura, I., Sasaoka, T., 2007. *Coenzyme Q10 prevents high glucose-induced oxidative stress in human umbilical vein endothelial cells.* European Journal of Pharmacology, 566, 1–10.

Tuder, R. M., Cool, C. D., Geraci, M. W., Wang, J., Abman, S. H., Wright, L., Badesch, D., Voelkel, N. F., 1999. *Prostacyclin synthase expression is decreased in lungs from patients with severe pulmonary hypertension*. American Journal of Respiratory and Critical Care Medicine, 159, 1925–1932.

Tueller *et al.* for the Swiss Society for Pulmonary Hypertension, 2008. *Epidemiology of pulmonary hypertension, new data from the Swiss registry*. Swiss Medical Weekly, 138, 379–384.

Ueda, H., Kawanishi, K., Moriyasu, M., 2004. *Effects of ellagic acid and 2-(2,3,6-trihydroxy-4-carboxyphenyl)ellagic acid on sorbitol accumulation in vitro and in vivo*. Biological and Pharmaceutical Bulletin, 27, 1584—1587.

Uzun, O., Balbay, O., Comunoglu, U., Yavuz, O., Annakkaya, A. N., Guler, S., Silan, C., Erbas, M., Arbak, P., 2006. *Hypobaric-hypoxia-induced*

pulmonary damage in rats ameliorated by antioxidant erdosteine. Acta Histochemica, 108, 59—68.

Valensi, P., Picard, S., 2011. *Lipids, lipid-lowering therapy and diabetes complications.* Diabetes & Metabolism, 37, 15–24.

Vasir, J. K., Labhasetwar, V., 2007. *Biodegradable nanoparticles for cytosolic delivery of therapeutics.* Advanced Drug Delivery Research, 59, 718.

Vincent, J. A., Ross, R. D., Kassab, J., Hsu, J. M., Pinsky, W. W., 1993. *Relation of elevated plasma endothelin in congenital heart disease to increased pulmonary blood flow.* American Journal of Cardiology, 71, 1204–1207.

Vogel, R. A., Corretti, M. C., Gellman, J., 1998. *Cholesterol, cholesterol lowering, and endothelial function.* Progress in Cardiovascular Diseases, 41, 117-136.

Wadsworth, R. M., Hoehn, T., McPhaden, A., Stiller, B., Preston, A., 2004. Upregulation of nitrotyrosine, a marker for peroxynitrite, in pulmonary arteries of pulmonary hypertensive patients. In: Daniel Henrion A, editor. 8th International Symposium on resistance arteries (June 20e23). Angers, France: KARGER; 2004. p. 85.

Wang, Y-J., Pan, M-H., Cheng, A-L., Lin, L-I., Ho, Y-S., Hsieh, C-Y., Lin, J-K., 1997. *Stability of curcumin in buffer solutions & characterization of its degradation products.* Journal of Pharmaceutical and Biomedical Analysis, 15, 1867-1876.

Wang, X. L., Rainwater, D. L., Mahaney, M. C., Stocker, R., 2004. *Cosupplementation with vitamin E and coenzyme Q10 reduces circulating markers of inflammation in baboons.* The American Journal of Clinical Nutrition, 80, 649–655.

Wedgwood, S., Black, S. M., 2005. *Endothelin-1 decreases endothelial NOS expression & activity through ETA receptor-mediated generation of hydrogen peroxide.* American Journal of Physiology Lung Cell Mol. Physiol., 288, L480-L487.

Westesen, K., Siekmann, B., 2001. *Particles with modified physicochemical properties, their preparation & uses.* US6197349.

Whitley, A.C., Stoner, G.D., Darby, M.V., Walle, T., 2003. *Intestinal epithelial cell accumulation of the cancer preventive polyphenol ellagic acid-extensive binding to protein & DNA.* Biochemical Pharmacology, 66, 907–915.

Witting, P. K., Pettersson, K., Letters, J., Stocker, R., 2000. *Anti-atherogenic effect of coenzyme Q10 in apolipoprotein E gene knockout mice.* Free Radical Biology & Medicine, 29, 295–305.

Xie, Z., Li, H., Wang, K., Lin, J., Wang, Q., Zhao, G., Jia, W., Zhang, Q., 2010. *Analysis of transcriptome and metabolome profiles alterations in fatty liver induced by high-fat diet in rat.* Metabolism. 59, 554-560.

Yamagishi, S., Nakamura, K., Matsui, T., Ueda, S., Fukami, K., Okuda, S., 2008. *Agents that block advanced glycation end product (AGE)–RAGE (receptor for AGEs)–oxidative stress system: a novel therapeutic strategy for diabetic vascular complications.* Expert Opinion on Investigational Drugs, 17, 983–996.

Yamagishi, S., Maeda, S., Matsui, T., Ueda, S., Fukami, K., Okuda, S., 2011. *Role of advanced glycation end products (AGEs) and oxidative stress in vascular complications in diabetes.* Biochimica et Biophysica Acta, Article in press.

Yang, R., Le, G., Li, A., Zheng, J., Shi, Y., 2006. *Effect of antioxidant capacity on blood lipid metabolism and lipoprotein lipase activity of rats fed a high-fat diet.* Nutrition, 22, 1185–1191.

Yang, R-L., Shi, Y-H., Hao, G., Li, W., Le, G-W., 2008. *Increasing oxidative stress with progressive hyperlipidemia in human: relation between malondialdehyde and atherogenic index.* Journal of Clinical Biochemistry and Nutrition, 43, 154–158.

Yi, X., Nickeleit, V., James, L. R., Maeda, N., 2011. *α-Lipoic acid protects diabetic apolipoprotein E-deficient mice from nephropathy.* Journal of Diabetes and Its Complications, 25, 193–201.

Yokoyama, H., Lingle, D. M., Crestanello, J. A., Kamelgard, J., Kott, B. R., Momeni, R., Millili, J., Mortensen, S. A., Whitman, G. J., 1996. *Coenzyme Q10 protects coronary endothelial function from ischemia reperfusion injury via an antioxidant effect.* Surgery, 120, 189-196.

Yoshihara, F., Nishikimi, T., Horio, T., Yutani, C., Takishita, S., Matsuo, H., Ohe, T., Kangawa, K., 1998. *Chronic infusion of adrenomedullin reduces pulmonary hypertension & lessens right ventricular hypertrophy in rats administered monocrotaline.* European Journal of Pharmacology, 355, 33–39.

Yu, Y-M., Chang, W-C., Wu, C-H., Chiang, S-Y., 2005. *Reduction of oxidative stress and apoptosis in hyperlipidemic rabbits by ellagic acid.* Journal of Nutritional Biochemistry, 16,675-681.

Zmitek, J., Zmitek, K., Pravst, I., 2008a. *Improving the bioavailability of CoQ_{10}, from theory to practice.* Agro Food Industry Hi-Tech, 19, 4-9.

Zmitek, J., Smidovnik, A., Fir, M., Prosek, M., Zmitek, K., Walczak, J., Pravst, I., 2008b. *Relative bioavailability of two forms of a novel water soluble CoQ10.* Annals of Nutrition and Metabolism, 52, 281-287.