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INVESTIGATIONS ON SCALE UP AND SHELF-STABILITY OF CURCUMIN ENCAPSULATED POLYMER NANOPARTICLES FOR TREATING INFLAMMATORY AND VASCULAR DISEASES

BY

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Copyright statement

Signed: *Date:*

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List of Publications

Research

C.N. Grama, V.P. Venkatpurwar, D.A. Lamprou and M. N. V. Ravi Kumar. Towards scale-up and regulatory shelf-stability testing of curcumin encapsulated polyester nanoparticles. ***Drug Deliv. Transl. Res***. 3, 286-293, 2013.

C. N. Grama, P. Suryanarayana, M. A. Patil, G. Raghu, M. N. V. Ravi Kumar and G. Bhanuprakash Reddy. Efficacy of biodegradable curcumin nanoparticles in delaying cataract in diabetic rat model. ***PLoS One*** (Revised and Submitted)

C. N. Grama, A. Ahmad S. Periyasamy-Thandavan, S. Fulzele, S. Ahmad, S. Siddiqui, Md Nasrul Hoda and M.N.V. Ravi Kumar. Evaluating the potential of nanoparticulate curcumin in acute ischemic stroke model. Stroke (In Preparation)

Reviews

C. N. Grama, D. D. Ankola and M. N. V. Ravi Kumar. Poly(lactide-co-glycolide) nanoparticles for peroral delivery of bioactives. ***Curr. Opin.Colloid Interface Sci.*** 16, 238-245, 2011.

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Abstract

Curcumin, a natural spice of Indian origin is the active principle present in the yellow spice turmeric. However, this molecule is under-utilized due to its poor *peroral* bioavailability. Very recently, we have demonstrated that encapsulation of curcumin in biodegradable nanoparticles improve oral bioavailability of curcumin. This study reports scale-up and shelf-stability of curcumin encapsulated poly (lactic acid-*co*glycolic acid) (PLGA) nanoparticles (nCUR). The nCUR were prepared by emulsification solvent evaporation/diffusion method and by varying the process parameters such as homogenisation duration we could successfully prepare large quantities nCUR. The particle size decreased as the homogenisation duration increased from 5 min to 30 min and the particles were spherical.

The particle characteristics of large scale preparation (particle size 288.7±3.4 nm and curcumin entrapment efficiency of 52.5 ±4.3%) were similar to those obtained by lab-scale preparation. The freeze dried nCUR were subjected to six-month ICH shelf-stability conditions suitable for room temperature and refrigerated storage. The nCUR were found to retain the stability over test period as determined by particle characteristics and curcumin integrity using AFM, zeta sizer and XRD analysis.

Curcumin has been shown to exhibit various pharmacological actions such as antioxidant, anti-inflammatory, antimicrobial, and anti-carcinogenic activities. Therefore, attempts were made to evaluate the potential of nCUR in models of diabetic cataract, stroke and cancer. In diabetic cataract model, oral administration of 2 mg/day nCUR was significantly more effective than curcumin in delaying cataract progression independent of its glucose lowering ability but with significant effect on aldose reductase pathway

reducing sorbitol levels and osmotic pressure. The nCUR was found to be very effective in acute ischemic stroke and the activity was mediated via prevention of oxidative stress, inflammation and MMPs over expression. However, nCUR was not as effective as reported in the literature in ovarian cancer xenograft, but a slight reduction towards 18-19 days post implantion but was not significant. On the other hand, nCUR was ineffective in B16 F0 orthotopic xenograft lung tumour model.

Together, this data indicate the potential of curcumin in treating a variety of diseases and nCUR was superior due to its ability to improve *peroral* bioavailability.

List of Abbreviations

ADI	average daily intake
AF	atrial fibrillations
AFM	Atomic Force Microscopy
AGEs	advanced glycosylation end products
AIFs	apoptosis-inducing factors
AO	Antioxidants
APN	adiponectin
AR	Aldose reductase
BLI	Bioluminescent Imaging
BSA	bovine serum albumin
CBF	cerebral blood flow
CCA	common carotid artery
CCL	Chemokine (C-C motif) ligand
CD4OL	CD4O ligand
CMC	Carboxymethyl cellulose
CML	N(epsilon)-(carboxymethyl)lysine
CRP	C-reactive protein
CSCs	cancer stem cells
CVA	cerebrovascular accident
CVD	cardiovascular diseases
CXCL	C-X-C motif chemokine
DCCT	Diabetes Control and Complications Trial
DHR123	dihydrorhodamine 123
DLS	dynamic light scattering
DM	diabetes mellitus

DMSO	dimethylsulphoxide
DNA	deoxyribose nucleic acid
DPP-IV	dipeptidyl peptidase-IV
DR	diabetic retinopathy
EB	Evan's blue
ECA	external carotid artery
ECL	enhanced chemiluminescence
EDIC	Epidemiology of Diabetes Interventions and Complications
EDTA	ethylene diamine tetra acetate
EPR	enhanced permeation and retention effect
FAS	fatty acid synthase
FBS	foetal bovine serum
FITC	Fluorescein isothiocyanate
GAPDH	glyceraldehyde-3 phosphate -dehydrogenase
GLUT2	glucose transporter 2
GPx	glutathione peroxidise
GSH	glutathione
H & E	Hematoxylin and eosin
H_2O_2	hydrogen peroxide
$HbA1_c$	glycated haemoglobin
HMW	high molecular weight
HPCs	hepatic stellate cells
HPLC	high pressure liquid chromatography
HPV	human papillomavirus
HSC	hematopoietic stem cell
hsCRP	high sensitive C- reactive protein
HSPs	heat shock proteins

ICA	internal carotid artery
ICAM	Intercellular adhesion molecule
ICH	International Conference of Harmonization
IL	Interleukin
iNOS	Inducible Nitric oxide synthase
IP	intraperitoneal
IS	Internal standard
IVIS	In vivo imaging system
KATP	ATP-sensitive potassium
LDL	Low-density lipoprotein
mAbs	monoclonal antibodies
MCAO	middle cerebral artery occlusion
MCP-1	Monocyte chemoattractant protein-1
MDA	malondialdehyde
MMPs	Matrix metalloproteinases
MRI	Magnetic Resonance Imaging
MSC	mesenchymal stem cells
NAD	nicotinamide adenine dinucleotide
NADPH	nicotineamide adenine dinucleotide phosphate-oxidase
nBLA	Blank nanoparticles
nCUR	curcumin encapsulated polyester nanoparticles
NF-κB	nuclear factor kappa beta
NO	nitric oxide
$NO^{\bullet}$	nitric monoxide
NO_3^-	peroxynitrite radical
$O_2^{\bullet -}$	superoxide
OGD	oxygen-glucose deprivation

$OH^{\bullet}$	hydroxyl
PBS	phosphate-buffered saline
PDGF	platelet-derived growth factor
PDI	polydispersity index
PKC	protein kinase C
PLGA	poly(lactide-co-glycolide)
PPARs	peroxisome proliferator-activated receptors nuclear
PPAR-γ	gamma PPAR
PVA	polyvinyl alcohol
PVDF	polyvinylidene difluoride
RAGE	receptor for AGEs
RES	reticuloendothelial system
RH	Relative humidity
RNS	reactive nitrogen species
RONS	Reactive oxygen and nitrogen species
ROS	reactive oxygen species
rpm	rotations per minute
RT PCR	Real time polymerase chain reaction
SD	Sprague Dawley
sd	Standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SLN	solid lipid nanoparticles
SOD	superoxide dismutase
STZ	streptozotocin
T1D	type 1 diabetes
T2D	type 2 diabetes
TBARS	thiobarbituric acid reacting substances

TF	Transcription factor
TME	tumour microenvironment
TNF-α	tumor necrosis factor-alpha
tPA	tissue plasminogen activator
TTC	triphenyltetrazolium chloride
UKPDS	United Kingdom Prospective Diabetes Study
VCAM	Vascular cell adhesion molecule
VEGF	vascular endothelial growth factor
XRD	X-ray diffraction

List of Symbols

%	Percentage
μ	Micro
μg	Microgram
α	Alpha
β	Beta
γ	Gamma
κ	Kappa
ml	Millilitre(s)
mm	Millimetre(s)
min	Minute(s)
kDa	Kilodalton
rpm	Revolutions per minute
g	acceleration due to earth's gravity
w/v	Weight by volume
°C	degrees celsius
h	hour
mBar	milliBar (pressure)
μl	Microlitre
kHz	Kilohertz
λmax	Lambda max
nm	Nanometre(s)
kV	Kilovolt(s)
mA	milliampere
min−1	Per minute
w/w	Weight by weight

au	Absorbance unit
μmol	Micromolar
λem	Fluorescence intensity

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1 GENERAL INTRODUCTION

Inflammation is a complex protective host reaction against injurious agents causing injury to the cells and can be classified as acute or chronic inflammation. Ironically, inflammation is also one of the most common means whereby our own tissues are injured. The two main components of inflammatory reactions are the vascular and cellular reactions which are mediated by circulating cells, plasma proteins and cells of surrounding connective tissues and vascular walls. The simultaneous occurrence of active inflammation, tissue destruction and attempts at repair can be termed as chronic inflammation (Kumar *et al*., 2007). The chronic inflammation results in progressive destruction of the tissue that compromises the survival of the organism as well as lead to a host of diseases (Fig 1).

Normally, on cellular aerobic metabolism, the mitochondria produce energy and free radicals which are unpaired electrons (Halliwell 1994) that plays an important role in various biological processes. The reactive oxygen species (ROS) are the most reactive among the free radicals and commonly reported ones are hydroxyl ($OH^{\bullet}$), superoxide ($0_2^{\bullet -}$), nitric monoxide ($NO^{\bullet}$) and hydrogen peroxide (H_2O_2). Despite regulating the signalling of mediators at moderate concentration, ROS can be threatening and can cause disturbance in the cellular constituents leading to various diseases (Adly, 2010). In chronic inflammatory process, oxidative stress, nitrosative stress and lipid peroxidation takes place with excess production of ROS and DNA-reactive aldehydes as seen in many diseases such as arthritis, cardiovascular diseases (CVD), diabetes mellitus (DM), cancer, stroke and other major disorders (Freidovich., 1999; Karthikeyan *et al*., 2011). It is still unclear whether the free radicals trigger the disease or if they are produced due to the disease or from the damaged tissue (Valko *et al*., 2007). There are several enzymatic systems

such as superoxide dismutase (SOD), glutathione peroxidise (GPx) and catalase in the body which interact with the free radicals safely and terminate further oxidation reactions (Adly, 2010) however; some reactive species escape the enzymatic degradation. Hence, antioxidant nutrients play an important role in maintaining and optimizing the performance of chain reactions and the immune system (Miller *et al*., 1993).

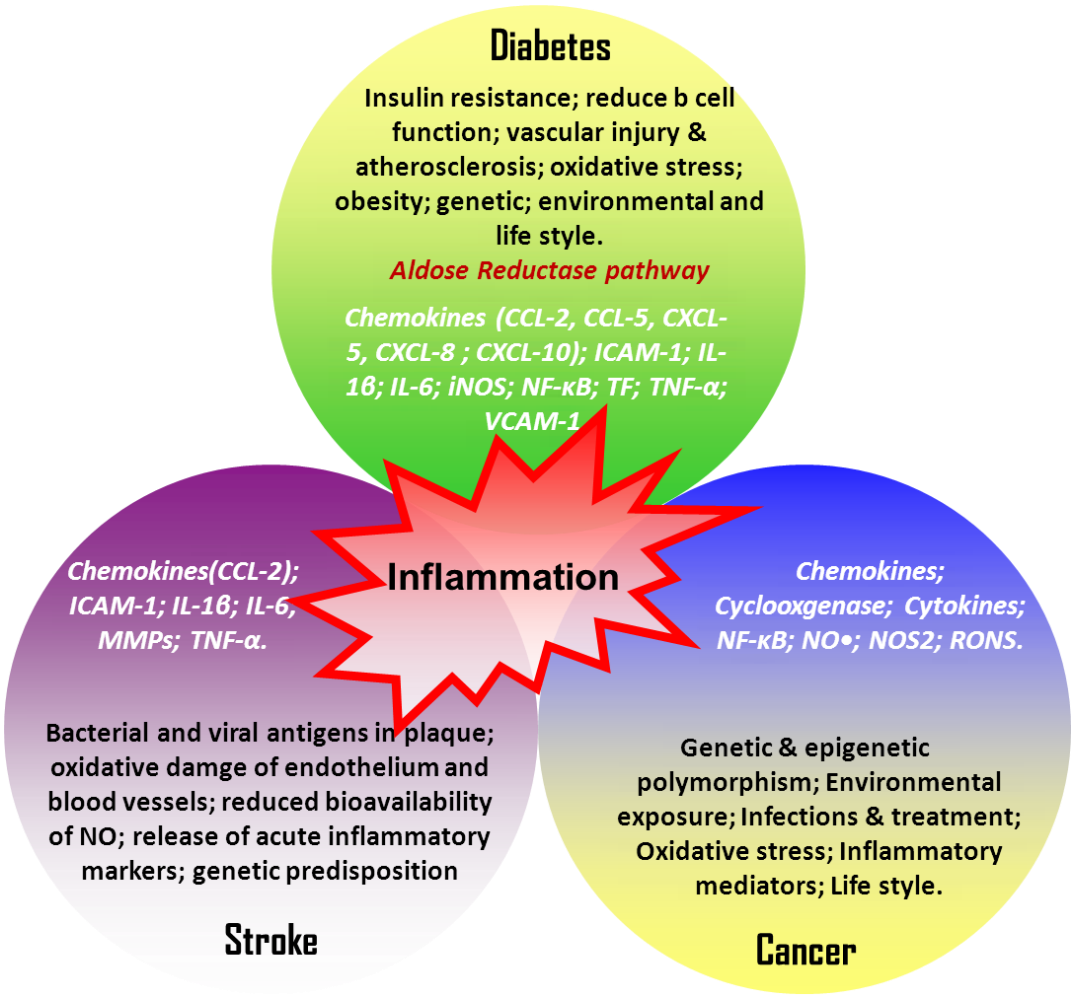

Figure 1.1 A schematic of inflammatory cascade in diabetes, stroke and cancer.

1.1 Role of inflammation in diabetes

Experimental and prospective studies have shown that type 2 diabetes (T2D) is a heterogeneous metabolic disorder with several different molecular, inflammatory, oxidative stress mechanisms (Östenson, 2001). Inflammation is induced at the time of stress due to environmental, psychosocial, behavioural, diet and individual factors contributing to the pathogenesis of T2D (Astrup & Finer, 2000). Chronic low-grade inflammation and innate immune activation has been closely studied since it was first proposed in 1997 in the pathogenesis of T2D (Pickup, 1997; Pickup *et al*., 2004). A chronic subclinical systemic and vascular inflammation occurs due to numerous mechanical and biochemical factors induced by pro-inflammatory mediators such as tumour necrosis factor (TNF), interleukin 6 (IL-6), and C-reactive protein (CRP) (Hardy *et al*., 2012) on the endothelium inhibiting the phosphorylation of the insulin receptors on tyrosine and diminishing the endothelial insulin response. When this process is enhanced due to insulin resistance, it leads to slow development of micro- and macrovascular complications (Hartge *et al*., 2007; Bertoni *et al*., 2010). The reports suggesting the role of plasma adiponectin (APN) levels in the pathogenesis of diabetic retinopathy (DR) is controversial (Yilmaz *et al*., 2004; Kato *et al*., 2008).

Several studies in experimental animals and diabetic patients have reported that an increase in blood glucose levels leads to development of oxidative stress producing free radicals in type 1 diabetes (T1D) and T2D (West 2000). During oxidative stress there is a constant imbalance between the ROS and reactive nitrogen species (RNS) production affecting both activity of cells and ability of cells to secrete therefore beta-cell function fails (Drews *et al*., 2010). Auto-oxidation of glucose (Wolff 1993), glycation of antioxidant enzymes

(Mullarkey *et al*., 1990), interaction between the glycated proteins and receptors (Schmidt *et al*., 1994) and ketosis (Jain *et al,* 1998) together leads to excessive radical production and forms an important role in the aetiology of DM. Also excessive nicotineamide adenine dinucleotide phosphate-oxidase (NADPH) present in various cell membranes produced by glucose metabolism stimulate cytochrome P450-like activity is responsible for generation of ROS (Jain 1989; Newsholme *et al*., 2009) and these reacted with cellular components causing irreversible modifications (Lyons, 1991). Polyol pathway, activation of protein kinase C (PKC) and increased formation of glucose-derived advanced glycosylation end products (AGEs) are other pathways identified in the aetiology of micro- and macrovascular complications of diabetes.

Aldose reductase (AR) pathway was the first pathway described with consumption of intracellular NADPH during reduction of glucose to sorbitol and then to fructose resulting in depletion of intracellular antioxidant enzymes such as glutathione (GSH) (Altan, 2003; Obrosova *et al*., 2005a, b). This in turn results in increased sensitivity of cells to oxidative and nitrosative injury and production of AGEs triggering inflammatory response and PKC pathway (Yan *et al*., 1994; Goldin *et al*., 2006). Interaction of AGEs with receptor for AGEs (RAGE) also results in production of cellular ROS (Basta *et al*., 2005; Cai *et al*., 2006). By inhibiting the formation of mitochondrial reactive species a number of pathways of T2D are normalised (Brownlee, 2001; Nishikawa *et al*., 2007).

The eye lenses also have antioxidant enzymes such as SOD, catalase (Hashim & Zarina, 2006), Paraoxonase (Hashim & Zarina, 2007) and GSH (Ganea & Harding, 2006) for protection against damage which showed compromised activity in subjects suffering from diabetic cataract (Hashim *et al*., 2009).

Earlier studies have implicated towards the convergence of many pathways and contributing to hyperglycemia induced cataract (Hashim & Zarina, 2006; 2007; 2011; 2012; Hashim *et al*., 2009). Very recently role of aqueous humor (AH) and APN was studied in the pathogenesis of proliferative diabetic retinopathy (Mao *et al*., 2012).

1.1.1 Treatment of diabetes

The main treatment goals of DM are to bring the glucose concentrations in blood as close to normal and to prevent and/or minimize the development of its complications (Holman *et al*., 2008). The standard therapeutic option knowing the etiopathology of T1D is replacement by different regimes of insulin injection. Alternative therapies such as pancreatic transplantation (Robertson *et al.,* 2004) and human islet transplantation (Shapiro *et al*., 2000) were associated with complications due to transplant procedure, long term use of immunosuppressant therapy and hunt for organ donors and transplant materials. Transplantation of hematopoietic stem cell (HSC) after high dose of immunosuppressant therapy to stop destruction of β-cells and replacement of damaged immune system by HSC showed promising results (Voltarelli *et al*., 2007). Although, the mesenchymal stem cells (MSC) therapies have provided promising results in animal models the oncogenic potential should be kept in mind (Vija *et al*., 2009).

In T2D, effective control of glucose concentrations in blood as well as blood pressure and lipids minimizes the chronic complications of diabetes. A combination of weight loss, exercise and diet control is the initial steps of management. When diabetes still remains uncontrolled, hypoglycemic drugs or even insulin are the next best options. It has been reported that exposure

of β-cells to constant hyperglycemic conditions can be shielded from impairment by introducing insulin at an earlier stage (Meneghini, 2009). A randomized study conducted by DCCT, 1993 (Diabetes Control and Complications Trial) and DCCT/EDIC, 2000 (the Epidemiology of Diabetes Interventions and Complications), showed diabetic complications such as retinopathy, nephropathy and neuropathy are significantly delayed by intensive therapy and strict control of blood glucose concentrations (Nathan *et al.*, 2005).

There are a number of oral agents for treatment of T2D (Guthrie, 2012). Depending on the mode of action anti-diabetic drugs can be categorized into agents that increase the secretion of insulin from the pancreas, decrease hepatic gluconeogenesis and increase the uptake of glucose by the periphery by increasing tissue sensitivity to insulin. Hypoglycemia is the most common complication in patients taking orals hypoglycemic agents mainly with sulfonylureas and also seen with insulin therapy (Lebovitz, 2011). Treatment of DM is now more diverse and has captured more attention than the conventional agents as these newer pharmacological agents such as exenatide, dipeptidyl peptidase-IV (DPP-IV) inhibitors and rimonabant target the key issues such as weight gain and hypoglycaemia (Srinivasan *et al*., 2008). There has been a considerable improvement in medical therapies for subjects with T2D where novel mechanism of action such as sodium-glucose co-transporter 2 inhibitors, the G-protein-coupled receptor agonists, and the balanced dual PPAR-α/γ agonists are being focused (Lebovitz, 2011). Promising compounds which directly act within the gastrointestinal tract lowering postprandial glucose excursion, activation of the brain D_2 dopamine receptors to lower plasma glucose levels and non-injectable preparations providing exogenous insulin which are the next generation

therapeutic options for T2D are being explored. Therefore, the recent technological developments in diabetes should aim at providing long lasting and effective treatment and have minimal side effects.

1.1.2 Diabetes and antioxidants

Antioxidants are defined as substances which inhibit the process of oxidation forming complex polymeric systems (Ho *et al.,* 1994) having diverse physiological role in protection of human body against damage by ROS (Lollinger, 1981). Recently, a variety of antioxidants have been studied for the treatment and prevention of a variety of diseases (Devadasu *et al*., 2006; Benfeito *et al*., 2013). The sources of oxidative stress in diabetes are enzymatic, non-enzymatic and mitochondrial pathways (Johansen *et al*., 2005). It has also been clear that antioxidants target mitochondrial ROS and other sources of ROS (Nishikawa *et al.*, 2007).

There are evidence suggesting vitamin E supplementation in rats and humans significantly reduced development of diabetes (Montonen *et al*., 2004). Supplementation of antioxidants such as ascorbic acid, alpha-tocopherol and alpha-lipoate shows beneficial effects with regards to oxidation of LDL, isoprostanes and release of superoxide (Vega-López *et al*., 2004). Combination of high doses of micronutrient antioxidant vitamins administered rather than single supplement is beneficial in management of DM and its complications (Opara, 2002; Aly and Mantawy, 2012). Supplementation of micronutrients such as vitamin C, D and E modified the innate immune response inhibiting the oxidative stress and subsequent signalling of the proinflammatory markers thereby delaying or preventing the disease process (Garcia-Bailo *et al*., 2011). Alpha-lipoic acid, a potent

antioxidant has shown to protect the β-cell function (Cummings *et al*., 2010; Yi *et al*., 2011). Although alpha-lipoic acids, vitamin E and omega-3 fatty acid differed in their effects by showing improvement in different parameters, combination of these can improve the sensitivity to insulin and metabolism of lipid (Udapa *et al*., 2012). Recently, anti-inflammatory activity of Emblica *officinalis Gaertn* leaf extract has been evaluated in a streptozotocin (STZ) induced rat model of T2D (Nain *et al*., 2012).

But, recent meta-analyses have shown high dose of vitamins used to augment the risk of numerous diseases offered no benefits in a certain portion of population (Miller et al., 2005).

1.1.3 Diabetes and curcumin

In recent years, curcumin has increasingly gained scientific interest and has been studied in diabetes and its complications such as retinopathy, neuropathy, nephropathy and hepatic fibrosis (Meng *et al*, 2013; Stefanska, 2012). Significant amount of research have been conducted to determine the mechanism of action by which curcumin supplementation prevent disease process in diabetes. Curcumin activated the β-cell functioning and protected it from apoptosis not only by decreasing ROS production *in vitro* (Meghana *et al*., 2007) but also by inducing heat-shock protein 70 during cryopreservation (Kanitkar and Bhonde, 2008). From previous studies, it is also know that when red blood cells were exposed to high blood glucose levels, curcumin treatment inhibited glycation of proteins, lipid peroxidation and generation of oxygen radical improving the protein levels and renal and hepatic function markers (Jain *et al*., 2006; Murugan & Pari, 2007). Plenty of reports have also suggested curcumin acts by exhibiting hypolipidemic activity

(Babu & Srinivasan, 1997; Murugan & Pari, 2006). The role of various inflammatory cytokines such as, NF-κb, TNF-α, PPAR-γ in conjugation with curcumin has been implicated. The antinociceptive activity of curcumin was as a result of inhibition of TNF-α and release of NO (Sharma *et al*., 2006; 2007).

Curcumin supplementation has shown to reduce T2D by decreasing superoxide production and inhibiting PKC (Rungseesantivanon *et al*., 2010). When curcumin was dosed to diabetic mouse models, pancreatic islets underwent neogenesis concluding that curcumin has the antidiabetic potential (Chanpoo *et al*., 2010). Two recent Egyptian studies investigated the use of curcumin in T2D (El-Azab *et al*., 2011; El-Moselhy*et al*., 2011). The treatment of curcumin for 16 weeks in rat model of diabetic retinopathy showed significant reduction in the retinal levels of pro-inflammatory cytokines, TNF- α and vascular endothelial growth factor (VEGF) preventing damage to endothelium and thickening of the capillary basement membrane. This study revealed curcumin treatment prevented structural degeneration and significantly reduced the induction of diabetic retinopathy (Gupta *et al*., 2011). An *in vitro* study on hepatic stellate cells culture (HPCs) treated with curcumin inhibited cell proliferation, collagen production, expression of genes and glucose transporter 2 (GLUT2) by activating peroxisome PPAR-γ and GSH synthesis (Lin & Chen, 2011). In another study, administration of curcumin in diabetic rats for 8 weeks inhibited the expression of VEGF lining the podocytes and renal tubules demonstrating the angiogenic potential of curcumin and maintenance of structure of the kidney in DM (Sawatpanich *et al*., 2010). Curcumin has been reported to prevent obesity by inhibiting fatty acid synthase (FAS) preventing 3T3-L1 cell differentiation and accumulation of lipids (Zhao *et al*., 2011).

1.2 Role of inflammation in stroke

The role of inflammation in the pathogenesis of stroke has not been fully understood. Cardiovascular risk factors such as hypercholesterolemia and hypertension with genetic predisposition and infection interact with one another directly or indirectly leading to the formation of atheromatous plaques in larger arteries and thrombosis in smaller arterioles (Perttu & Grau 2003; De Roos *et al.,* 2005). These atherosclerotic lesions are immune mediated inflammatory response and mature through release of several factors such as cytokines, chemokine's and growth factors increasing the risk of embolism and stroke (Perttu & Grau 2003). During inflammation, chemokine's not only regulate transportation of leukocyte but also activates platelets and contribute to the progression of atherosclerosis (Kowalska *et al*., 2000; Abi- Younes *et al*., 2000). Monocyte chemoattractant protein-1 (MCP-1) was found to overexpress in atherosclerotic lesions (Nelken *et al*., 1991; Yla-Herttuala *et al*., 1991). CD4O ligand (CD4OL) expressed on the surface of platelets adhere to the cell membrane producing soluble fragments, sCD40L activating the Matrix metalloprotinease (MMPs) and procoagulant activity playing a major role in atherothrombosis in ischaemic stroke (Inwald *et al*., 2003; Garlichs *et al*., 2003). Cerebral ischaemia follows multiple cascades of apoptosis where the mitochondrial levels of anti-apoptotic Bcl-2 protein decreased with subsequent increase in cytosolic translocation of cytochrome c and increased caspase activation causing fragmentation of DNA (Zhao et al., 2010). It was also noted serum high sensitive C- reactive protein (hsCRP) is not only a marker but also contributes to the development and instability of atheroma (Ding *et al*., 2008).

Apart from damage to the brain tissue, cerebral ischaemia also alters the peripheral immune system. The inflammatory cells play a multiplex role that demonstrates a beneficial and detrimental response after ischaemic stroke. There is infiltration of various inflammatory cells such as neutrophils, monocytes, T-cells and microglial cell and loss of lymphocytes. Accumulation of T cells in the ischemic brain effect the progression of tissue inflammation and injury (Yilmaz et al., 2006). Microglial cells are known to exert both neuroprotective and neurotoxic effect. Different subtypes of T cells have been involved in the pathogenesis of stroke (Jin *et al.,* 2010).

After cerebral-reperfusion ischaemia, several components of ROS such as $OH^{\bullet}$, $0_2^{-\bullet}$, H_2O_2 and peroxynitrite radical (NO_3^-) are generated impairing the mitochondrial function and damaging the cellular membrane, proteins and DNA of neurons (Sims *et al*., 2000). Cerebral ischaemia also leads to disintegration and/or aggregation of cellular proteins such as SOD and GPx resulting in the endogenous defence mechanism failure against ROS (Davies 1987; Davies and Delsignore 1987; Davies *et al*., 1987a; Davies *et al*., 1987b).

The NO_3^- is a toxic anion formed by the conjugation of nitric oxide (NO) and superoxide anion($O_2^{\bullet -}$) (Dawson and Dawson., 2004) interfering with key enzymes of the Krebs cycle, metabolism of Ca^+ in mitochondria and the mitochondrial respiratory chain causing endothelial injury (Gobbel *et al*., 1997; Shibuta *et al*., 1998). Following cerebral ischaemia, astrocytes produce inducible NO synthase (iNOS) (Khan *et al*., 2005) which are NF-κB activation dependent (Togashi *et al*., 1997) resulting in over expression of several cytokines TNF, IL-12, IL-6 and IL-8 (Kröncke *et al*., 1998) eventually responsible for NO_3^- formation and breakdown of blood brain barrier (Jiang *et al*., 2007).

It has also been reported the role of homocysteine and heat shock proteins (HSPs) as risk factors acting in association with the conventional risk factors and genetic predispositions in the pathogenicity of stroke (Banecka-Majkutewicz *et al*., 2012).

1.2.1 Treatment of stroke

The initial definitive treatment for stroke focuses on restoring blood flow in ischaemic stroke and control of bleeding for haemorrhagic stroke. In ischaemic stroke, the block can be removed either by giving thrombolytic agents or mechanically by thrombectomy. In haemorrhagic stroke, to determine and evaluate the cause of bleeding an emergency surgery, craniotomy is often needed although many may not require surgery. Drug delivery to brain is challenging where the drug should possess both the CNS therapeutic effect and has the ability to cross the blood brain barrier (Barchet & Amiji, 2009).

In ischaemic strokes, the blockage is removed chemically by using tissue plasminogen activator (tPA) or recombinant tissue plasminogen activator (rtPA) intravenously. A pooled meta-analysis study analysed the relation between onset of stroke to start of treatment (OTT) and showed favourable outcome and benefits when the treatment with thrombolysis was started within 4.5 h from stroke onset (Lees *et al*., 2010). Intracerebral haemorrhage, high cost, lack of benefits and narrow window of opportunity was considered to be the main reason for restricted use of tPA by the clinicians (Brown *et al*., 2005).

Several clinical studies investigated the efficiency of antiplatelet therapy in the treatment of stroke. Clopidogrel versus aspirin in patients at risk of

ischaemic events (CAPRIE) trial supported the beneficiary effect of clopidogrel over aspirin in secondary stroke prevention (Barer, 1997). The European/Australasian stroke prevention in reversible ischaemia Trial (ESPIRIT) and second European stroke prevention study (ESPS-2) showed combination therapy of antiplatelet therapy had better outcome than with single therapy though the management of atherothrombosis with clopidogrel in high-risk patients (MATCH) trial showed no difference in the reduction of vascular event or bleeding in high risk patients (Gorelick, 2008). The clopidogrel for high atherothrombotic risk and ischaemic stabilization, management, and avoidance (CHARISMA) trial did show the benefit with dual antiplatelet therapy compared to aspirin alone (Gorelick, 2008). The superiority of clopidogrel therapy against aspirin and extended release dipyridamole (ER-DP) was the outcome studied by the prevention regimen showed for effectively avoiding second strokes (PRoFESS) study but was preferred to aspirin due to patient compliance and less bleeding (Selim, 2009). Administering aspirin within 48 h of onset of symptoms and continuing it for 2 – 4 weeks reduced the risk of recurrent stroke (the International stroke trial and the Chinese acute stroke trial) (Chen et al., 2000). A new oral anticoagulant dabigatran showed advantages over warfarin in patients with atrial fibrillations (AF) (the randomized evaluation of long-term anticoagulation therapy, RE-LY) (Chaturvedi &Bhattacharya, 2011).

Hence from these clinical studies it appears that mono or dual antiplatelet therapy are the choice of treatments in patients without any source of cardio embolism while warfarin and direct thrombin inhibitors are effective in patients with risk factors such as AF. Genetics also plays an important role in the etiopathogenesis of stroke and recently several gene based therapies have

been evaluated and offered promising results in experimental models and not yet been developed in clinical practice (Markus 2012).

Multidisciplinary involvement by the physiotherapists, speech and language therapists, occupational therapists, ophthalmologists, pharmacists, psychologists, as well as physicians and nurses trained in rehabilitation medicine plays a crucial role in stroke treatment (Monaghan *et al.,* 2005).

Prevention of stroke can be either primary or secondary (Goldstein *et al*., 2011). The risk factors such as healthy diet, exercising regularly and life style changes such as cessation of smoking and reduction of alcohols intake are within our control and should be looked into and be given more attention.

1.2.2 Stroke and antioxidants

Free radicals developed during cerebral ischemia contribute significantly to brain damage. Hence, in the last few decades lot of interest has been dedicated towards defining the role of antioxidant compounds in stroke (Ascherio, 2000; Slemmer *et al*., 2008).

Melatonin, widely produced endogenously has been explored for free radical scavenging properties. Its capability to cross the blood brain barrier (BBB) with ease and the invulnerability even at high doses has proved to be advantageous (Reiter *et al*., 1994). Number of pathways has been described how melatonin acts as an antioxidant in brain (Gupta *et al*., 2003). The neurological role of berberine, which is an isoquinoline alkaloid found in medicinal plants was studied *in vitro* and *in vivo* (after 48 h of middle cerebral artery occlusion-MCAO). Berberine inhibited the release of cytochrome *c* and apoptosis-inducing factors (AIFs) from mitochondria when triggered by oxygen-glucose deprivation (OGD) - induced injury (Zhou *et al*.,

2008). On the other hand there were reports/meta-analysis that are quite disappointing, eg. Thirteen randomised control trials with 166,282 participants in total were analysed showing no significant benefits of vitamin E supplementation in the prevention of stroke (Bin *et al*., 2011). Fruits and vegetables are rich source of flavonoids/polyphenols and are presumed to mediate neuroprotective effect by antioxidant property (Slemmer *et al*., 2008). Several population based studies have been conducted that offered limited protection in stroke and this limited success is related to the difficulty in translation from rodents to humans due to differences in neuroanatomical behaviour while the potency of the compounds itself is questionable (Cherubini *et al*., 2008).

1.2.3 Stroke and curcumin

Based on the *in vitro* studies and preclinical data curcumin is known to have a diverse pharmacological activity and very little is known about its neuroprotective mechanism against cerebral ischemia (Wang *et al*., 2005). Following focal cerebral ischemic injury in rats, the volume of the infarct was decreased and better neurological outcome was observed either through antiapoptotic mechanism or by decreasing oxidative stress (Zhao *et al*., 2010). Stimulation of neurogenesis and neuroplasticity (Kim *et al*., 2008) and replacement of injured neuronal circuits (Anderson *et al*., 2007) by curcumin were other modes of action following neuropathy injury. Recently, differential distribution of curcumin formulated in liposomes or polymer nanoparticles was observed in specific brain sites and the curcumin concentrations found was observed appropriate for antioxidant, anti-inflammatory, neurogenesis and neuroplasticity activity (Chiu *et al*., 2011). A

novel multi-target polyphenolic curcuminoid, CNB-001 was identified by Schubert and colleagues (Chen *et al.*, 2011). This was considered to be a better choice than curcumin because of its abilility to penetrate across the BBB and the stimulation of several pathways promoting neuronal repair and survival (Lapchak, 2011). Overall, curcumin seems to play a potential role in stroke prevention by its antioxidant, anti-inflammatory, antilipemic or antiaggregant property (Ovbiagele, 2008).

1.3 Role of inflammation in cancer

The notion of role of inflammation in cancer was first hypothesised by Dr Rudolph Virchow who discovered the presence of leucocytes in cancer tissues in 1863.. However, when the infection does not subside and chronic inflammation sets in, the disease process becomes harmful impacting directly on mutation or indirectly through molecular effects at various steps of cell growth causing neoplasia (Coussens & Werb, 2002, Mantovani, 2005). It is well known that inflammation is a major risk factor in most of the cancers. Some of the risk factors causing inflammation leading to cancer are tobacco smoking, asbestos, human papillomavirus (HPV), *Helicobacter pylori*, Hepatitis B & C virus, Epstein-Barr virus and stress (Shacter & Weitzman 2002).

Due to chronic inflammation, high levels of ROS and NO compounds intermediates are produced changing the transduction cascades and transcription factors (NF-κB) through number of cascades initiating carcinogenesis (Lonkar & Dedon, 2009; Schetter *et al*., 2010). Expression of pro-inflammatory cytokines such as TNF-α, AP-1, IL-1 and IL-6 enhanced

under chronic inflammatory conditions mediate different pathways and promote tumorigenesis (Wu & Zhou, 2010; Grivennikov *et al*., 2010).

Role of overexpression of chemokines, cyclooxygenase, lipooxygenase and MMPs in variety of cancers has been widely reported (Goulet *et al*., 2003; Ye *et al*., 2004; Chang *et al*., 2005; Ii *et al*., 2006). Despite these reports, there has also been conflicting evidence suggesting blockage to these inflammatory pathways can be harmful and have a negative effect where the inflammatory mediator promote tumorigenesis under certain conditions (Aggarwal 2006).

1.3.1 Treatment of cancer

In cancer, the ultimate goal in treating patients is to control the tumour growth and spread of the disease. Although we made a significant progress in understanding the pathogenesis and treatment of cancer, a complete cure has not been discovered yet while the current conventional treatment regimen to treat cancer has not changed much since last four decades.

Cancer is a complex disease process and there is no single treatment available. The management depends on the site and staging of tumour (Tumour node and metastasis) and the general condition of the subject. The conventional treatment approaches are chemotherapy, surgery and radiotherapy either singly or in combination of other treatment modalities. Though surgical removal is the most effective treatment when compared to any other individual forms of cancer therapy, the completeness of resection has been questioned (Ditta *et al.,* 2012) and some tumour masses are difficult to remove as they are embedded deeply in unreachable sites of the body (Parissis & Young, 2010).The major drawback of the chemotherapy is the cancer cells received insufficient amount of drug and affected the normal

cells in their path (Kakde *et al.*, 2011). Other disadvantages were altered biotransformation and bio-distribution, development of resistance to drugs, toxicity and problems with drug clearance (Stavrovskaya 2002; Kakde *et al.*, 2011). The radiation used in management of cancer not only kills the cancer cells but also damage normal cells (Veldeman *et al.*, 2008).

Recently much of the research has been focussed on targeted treatment and delivery such as targeted delivery of small molecules, discovery of new antibodies, viral therapy and antiangiogenics. These techniques overcome the problems associated with conventional chemotherapy (Aina *et al*., 2002; Dorsam & Gutkind, 2007; Meng *et al*., 2012).

Significant efforts are in place to improve the delivery of small molecules to cancer tissues/cells sparing non-target organs and killing cancer cells. The delivery systems are believed to reside in the systemic circulation with minimal drug release occurring for extended period by avoiding reticuloendothelial system (RES) and increasing their chances to localise in the tumour tissues by enhanced permeation and retention effect (EPR) (Sledge & Miller, 2003).

The first successful targeted therapy was Trastuzumab that acts by degrading and inducing antibody dependent cytotoxic activity of ErbB2 expression. Other therapies such as monoclonal antibodies (mAbs) and small molecule inhibitors were developed to target new vessel formation, pro-growth signal amplification and transmission, cell cycle activation or DNA replication (Urruticoechea *et al*., 2010). However, the antibody based immunotoxins targeting the mABs did not succeed beyond phase *I* clinical trials due to lack of efficacy and undesired side effects (Becker & Benhar, 2012).

Recently, a wide variety of viruses have been tried for cancer therapy where the virus targets the cancer cell and the virus replicates infecting and destroying the cancer cells (Urruticoechea *et al*., 2010).

Angiogenesis is the growth of vasculature and this is regulated by several cytokines such as IL-8, VEGF,platelet-derived growth factor (PDGF) and many more but the most potent of them all is VEGF (Dvorak *et al*., 1995). The balance between the proangiogenic and antiangiogenic factors released by the tumour and host cells regulates the process of angiogenesis. Currently many antiangiogenic compounds are being investigated that target either the VEGF or their inhibiting receptors (Rosen, 2005).

Convincing initial clinical trial results of survivin are considered to be the next new targets for anticancer therapy. This compound is mostly upregulated in the malignant cancer tissues (Duffy *et al*., 2007) and rarely expressed in normal tissue making themselves easy to be targeted and promote antiangiogenesis (Bergers and Benjamin, 2003).

Research on eradication of cancer stem cells (CSCs) which is believed to be the root of origin of cancer is still in the preliminary stage and considered to be target for cancer therapy and management (Yi *et al*., 2013). Hence, this basic change in idea of understanding the origin of CSCs, its molecular profile and interaction with the tumour microenvironment (TME) would improve therapeutic outcome.

1.3.2 Cancer and antioxidants

The main source of antioxidants come from diet and is available as vitamin and minerals. They defend cells from damage from unstable molecules known as free radicals that interfere with immune system causing cancer

(Fanga et al., 2002). With respect to the mechanism of their action they can be classified as enzymatic and non-enzymatic groups (Sies, 1993). Several large scale clinical trials for cancers were examined and reached different conclusions (Omenn *et al.,* 1994; Hennekens *et al.,* 1996; Lee *et al.,* 1999).

Oxidative stress and inflammation are interlinked (Bruce et al., 2000). There are strong evidences supporting the role of oxidative stress leading to carcinogenesis (Miranda et al., 2011). They not only attack and damage the nuclear and mitochondrial component of the cell (Dizdaroglu et al., 2002) but also attack other cellular components like lipids (Dizdaroglu et al., 2002; Marnett, 1999) and proteins (Stadtman, 1992) and cause oxidation. Some antioxidants act as oxidative molecules at certain concentration (Badajatia et al., 2010). Literature evidences suggests that in cancer there is an increased oxidative stress which results in lower antioxidant levels, where supplementation with antioxidants can be beneficial (Sharma et al., 2009; Klarod et al., 2011).

In vitro and in vivo studies have shown that intake of antioxidants can prevent cancer in animals (Borek, 2004) and reduce risks of certain cancers (van Gils *et al.,* 2005). There are several reports showing the potential benefits of antioxidants as adjuvants in cancer therapy by their ability to cause cancer cell apoptosis both in vitro and in vivo and inhibit tumour metastasis (Zimmermann et al., 2001), resisting the growth of tumour by their angiostatic factor (Ashino *et al.,* 2003) and have shown promising data in reducing side effect associated with conventional radiation therapy (Kennedy et al., 2001). Various dietary antioxidants have been investigated suggesting their ability to prevent, or even treat cancer (Khan *et al.,* 2008).

Though the antioxidants found benefits in a variety of cancers in preclinical setting, the success was not translated to the clinic and this failure could be due to a variety of factors that include but not limited to wrong choice of subjects, the wrong combination of antioxidants, the dose, the duration or inappropriate formulation.

1.3.3 Cancer and curcumin

Curcumin is known to target multiple pathways rendering its antioxidant, antiseptic, anti-inflammatory, antimalarial and analgesic properties that offer benefits in a variety of pathological conditions (Aggarwal *et al*., 2007; Masuda *et al*., 2001).

The anti-inflammatory property of curcumin is by inhibiting NF-κB which in turn decrease the production of inflammatory markers and suppress COX-2 and iNOS production (Singh & Aggarwal, 1995; Plummer *et al*., 1999). Curcumin is known to target several stages of carcinogenesis–tumor growth, angiogenesis and metastasis by affecting the phase I and phase II enzymes of cytochrome p450 enzyme system (Thapliyal & Maru, 2001). Curcumin activates caspase-8 indirectly activating poly ADP ribose polymerase (PARP) thus inducing apoptosis of tumor cells (Wang *et al*., 1995). The other type of cell death called autophagy is still being studied for chemoresistance in certain cancers (Górka *et al*., 2005). Curcumin has demonstrated its potential in regulating pro-angiogenic (Yoysungnoen *et al*., 2006) and anti-angiogenic effects (Perry *et al*., 2010).

Curcumin is water insoluble and exhibits poor *peroral* bioavailability (Anand *et al*., 2007) which is considered to be the major reason hampering its clinical acceptability. Several attempts were made to formulate curcumin to

overcome the bioavailability problems, however, these preparations are still in their early stage of development (Liu *et al.,* 2012). Pre-treatment with nanoparticulate curcumin enhanced chemo/radio sensitization in *in vitro* ovarian cancer cells by down regulating the expression of pro-survival proteins and increase the apoptosis of cancer cells (Murali *et al*., 2010). Another study conducted on prostatic cancer cell lines using curcumin loaded nanospheres showed controlled release of curcumin with increased uptake (Mukerjee & Vishwanatha, 2009).

1.4 Curcumin clinical trials

There are about 73 clinical trials using curcumin reported by National Institutes of Health (NIH) in wide variety of diseases that are completed, stopped, in their early phase or currently recruiting. The clinical outcomes with curcumin were not so promising but the general notion is that curcumin is well tolerated even at very high doses (eg 8g/day) however, there were reports opposing such claims (Burgos-Morón *et al.,* 2010). The activity of curcumin has been studied on variety of inflammatory conditions including chronic anterior uveitis (Lal *et al*., 1999), idiopathic inflammatory pseudotumors (Lal *et al*., 2000), pancreatic cancer (Dhillon *et al*., 2006), post-operative infection (Satoskar *et al*., 1986) and cancerous lesions (Kuttan *et al*., 1987) in humans and reported. Apart from safety and the mode of action of curcumin, the efficacy was also assessed during clinical trials. Very low bioavailability of oral curcumin due to first pass metabolism, poor solubility and instability could be some of the reasons for its failure in clinical use (Wang *et al*., 1997). This was seen in both preclinical models (Perkins *et al*., 2002; Holder *et al*., 1978) and humans (Sharma *et al*., 2001; Cheng *et al*., 2001;

Satoskar *et al*., 1986; Shoba *et al*., 1998).

Curcumin is isolated from a variety of plant species such as *Curcuma aromatica*, *Curcuma phaeocalis* (Tohda *et al*., 2006) and mainly from *Curcuma longa* however their content varies and hence it is difficult for one to point out the actual amount of curcumin used in the study. Also curcumin exists in several forms which exhibit different potencies making it difficult to interpret the literature (Ruby *et al*., 1995).

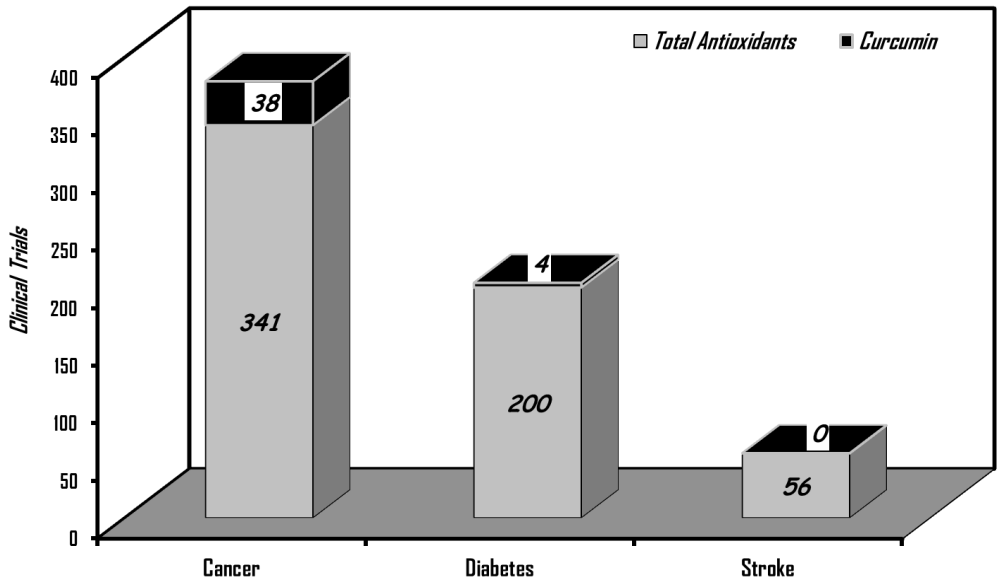

Figure 1.2 The number clinical trials in cancer, diabetes and stroke using antioxidants/curcumin (overall trails AO=2091 and curcumin=73).

Apart from curcumin, there is a significant amount of work being conducted using wide variety of antioxidants. Currently, there are about 2091 human trials on antioxidants (eg vitamin E, curcumin, coenzyme Q10, selenium etc) as therapeutic agents (www.clinicaltrials.gov accessed Feb 2013) in various diseases. Despite continuing research these antioxidants as anti-cancer therapeutic agents seems to be far from reality (Goodman *et al.*, 2011). Many factors contributing to the failure can be explained due to inappropriate

selection of the agent due to lack of understanding, the dosage and duration of the agent as opposed to dietary and the number of antioxidants tested upto date.

1.5 Advanced drug delivery systems

The ideal drug delivery system should be safe and facilitate the release of the encapsulated active compound at the site of action, thereby minimising the adverse effects if any, of the encapsulated active. Such delivery systems by virtue of their ability to sustain or control the release of encapsulated active are expected to reduce the dose and improve the efficacy by addressing some or all the issues associated with the active, such as poor solubility, poor permeability, rapid elimination, extensive metabolism (Bhardwaj *et al*., 2005). However, the use of delivery systems to address the inherent problems of antioxidants has not gained much interest as these compounds are marketed as supplements without therapeutic claims, so less invested and investigated (Kumar 2012).

Polymer nanoparticles have been extensively explored for drug delivery (Bala *et al.,* 2004, Grama *et al*., 2011). The drug molecule incorporated is protected from gut metabolism and gastrointestinal degradation after oral administration and hence the stability of the drug is achieved. Though the nanoparticles are believed to be absorbed intact and release the drugs in the systemic circulation no exact mechanism was put forward. However, it is believed that the absorption is predominantly through the intestinal lymphatic system (Hussain *et al*. 2001). The polymer nanoparticles are capable of delivering a wide range of bioactives improving the performance and minimizing the side effects (Grama *et al*., 2011; Bhardwaj *et al.,* 2005).

1.5.1 Literature review of curcumin delivery systems

Curcumin is known for poor systemic bioavailability after oral dosing because of extensive conjugation and reduction and rapid elimination (Shobha *et al.,* 1998; Sharma *et al.,* 2001, 2007). Therefore, research efforts are concentrated on improving the bioavailability, solubility and pharmacokinetic properties by designing and developing various types of curcumin formulations (Anand *et al.,* 2007; Yallapu *et al.,* 2012). The developments include self emulsifying systems (Setthacheewakul *et al.*, 2010) and conjugates (Kumar *et al.,* 2000; Manju & Sreenivasan, 2011) that enhanced the stability and poor aqueous solubility of curcumin. Recently, curcumin loaded PLGA nanospheres were formulated with success yeilding high efficious effects on many cell lines (Mukerjee & Viswanatha, 2009).The solid lipid curcumin NPs enhanced the bioavailability by increasing it absorption (Kakkar *et al.,* 2011). Among these the most widespread formulation of curcumin is the polymeric NPs and has been extensively used in chemoprevention (Shahani & Panyam, 2011; Tsai *et al.,* 2011), while other approaches such as lipidic systems (Pawar *et al.,* 2012) and polymer hydrogel nanoparticles (Dandekar *et al.,* 2010) are being investigated. Though the overall efforts of improving oral bioavailability have largely been successful, it is important to understand if these improvements in bioavailability results in therapeutic efficacy. Further, it is equally important to have a right model to investigate the therapeutic efficacy (Devadasu *et al.*, 2012). Though curcumin is found to be very effective *in vitro* and in a variety of preclinical models its dose varies widely for different aliments which calls for better delivery aids that fulfils the requirement.

The overall objective of this dissertation is to develop curcumin encapsulated nanoparticles and to test their ability to treat three different diseases characterised by inflammation. The doses used in these studies are similar or less than those reported in the literature for similar conditions, namely diabetes [10-75 mg/Kg] (Kumar *et al.,* 2005) stroke [100-300 mg/Kg] (Dohare *et al.,* 2008) and cancer [500 mg/Kg] (Lin *et al.,* 2007). The objective will be achieved by the following specific aims that are presented as chapters 2-5.

(1) Explore the possibilities of scale-up and shelf-stability of curcumin encapsulated nanoparticles.

(2) Evaluate the therapeutic potential of curcumin encapsulated nanoparticles in models of diabetic cataract, stroke and cancer.

2 SCALE-UP AND REGULATORY SHELF-STABILITY TESTING OF CURCUMIN ENCAPSULATED POLYMERIC NANOPARTICLES (nCUR)

2.1 Introduction

Polymer nanoparticles offer good flexibility with respect to the choice of bioactives ranging from water soluble to water insoluble and low to high molecular weight compounds that can be homogeneously distributed within the matrix or encapsulated in the particle core, though platform technologies remains elusive, research persists (Grama *et al.,* 2011; Devadasu *et al*., 2013). The development of drug encapsulated nanoparticles using preformed polymers involve emulsion techniques using organic solvents and surfactants that are currently made in small batch sizes of 50-100 mg polymer. It is a general perception that nanoparticulate formulations are believed to reduce the required dose by improving the bioavailability, though a cut off is not known in terms of the maximum dose that can be delivered by these nanoparticles. On the other hand while re-formulating any bioactive such as nanoparticles, the initial drug loading and the entrapment efficiency achieved has significant influence on the overall biological response which is not considered as an important factor (Ankola *et al*., 2010; 2011). This becomes all the more important with molecules like curcumin that is used as high as 12 g/day in the clinical trials (Gupta *et al*., 2013) and 500 mg/kg/day in preclinical studies (Lin *et al*., 2007).

Significant body of literature is available on curcumin's anti-inflammatory potential (Basnet & Skalko-Basnet, 2011) however, not much research is performed on formulating curucmin for improved performance and nanoparticulate approach seems to offer some hope of minimising the dose

offering a feasible dosage regimen (Devadasu *et al.,* 2006; Grama *et al.,* 2011; Yallapu *et al.,* 2012). In spite of the progress made in fundamental understanding of nanoparticulate formulations, the scale-up and shelf-stability of the developed formulations are still concerns, which we attempt to address in this chapter.

2.1.1 Preparation and scale-up

The most widely used method for preparation of nanoparticles is emulsion-diffusion-evaporation method (Hariharan *et al*., 2006). We used Poly (lactide-co-glycolide) (PLGA, 50:50) for preparation of nanoparticles which is a copolymer of lactic acid and glycolic acid available in different grades. PLGA is biocompatible and has ability to tailor the release properties and the degradation products *in vivo* are eliminated as CO_2 and water through Krebs cycle (Makadia and Siegel, 2011).Various studies have shown that the size, shape and surface chemistry of the nanoparticles not only plays a key role but also have a great impact on their cellular uptake, toxicity, interaction with plasma proteins and molecular response *in vivo* (Aggarwal *et al*., 2009; He *et al.,* 2010) However, majority of these studies are done under controlled environment using model particles, therefore it is difficult to extrapolate this information to realistic drug carriers and doses required for different ailments (Devadasu *et al.,* 2013). The nanoparticles size in principle can be controlled by the nature of surfactant, its concentration, organic solvent, its volume and homogenisation speed used in the preparation. For this work, we employed emulsion-diffusion-evaporation method for the preparation of curcumin encapsulated nanoparticles using PLGA with slight modifications

(Shaikh *et al*., 2009; Devadasu *et al.,* 2011) and most widely used surfactant, PVA due to low toxicity (DeMerlis & Schoneker, 2003).

Depending on the class of solvents and nature of the surfactant used for preparation of nanoparticles it is important to quantify the residual organic solvent and stabilisers to fulfil the regulatory requirements while the un-entrapped drug could interfere in processing of the dosage form as well as release, which need to be removed by purification process such as dialysis, filtration or centrifugation (Sahoo *et al*., 2002; Italia *et al.,* 2011). All the purification steps have been quite successful at lab scale production, such as dialysis and centrifugation (Italia *et al.,* 2011), gel filtration (Beck *et al.,* 1990), and occasionally cross-flow filtration (Quintanar-Guerrero *et al.,* 1998). Of all the methods centrifugation is routinely used in lab-scale though its success at large scale production remains to be studied (Yu *et al.,* 2006). Hence, the purification process plays a vital part in the preparation of large scale nanoparticles.

The preparation of nanoparticles in a larger scale has proved to be very challenging as majority of the work in the area is done at an academic level while very little efforts on scale-up and purification (Colombo *et al.,* 2001; Galindo-Rodríguez *et al.,* 2005). On a larger scale, there are many process limitations that are not apparent on laboratory scale. Large scale-up production of solid lipid nanoparticles (SLN) has been reported without any problems due to the availability of the homogenization lines in industry (Mehnert & Mäder 2001; Muller *et al.,* 2000).

2.1.2 Stability of nanoparticles

The ability to use nanoparticles in various dosage forms such as oral, parental, dermal, pulmonary and ocular is unique (Chavhan *et al*., 2011) but the most challenging and key issue during the process of formulation development is the shelf-stability of nanoparticles as such and the encapsulated bioactive.

Issues relating to stability of nanoparticles could arise anywhere in the process starting from manufacturing to shipping and storage of the product under the influence of factors such as temperature, humidity and light. The most common pathway of degradation is the erosion of the backbone of the polymer and thereby leading to breaking of the ester bonds causing rapid degradation of nanoparticles (Jain 2000). Drug nanoparticles, particularly those made using polymers that are hydrolysable usually have good storage stability as solids compared to suspension forms (Jain 2000).

The most common stability issues encountered with nanoparticles are aggregate formation, sedimentation, change in the crystalline state and crystal formation leading to different effects irrespective of the route they were administered (Wu *et al.,* 2011). For example, in pulmonary delivery, the site is affected and amount of drug delivered is decreased while in the intravenous route, there is obstruction in flow due to blocked capillaries.

The use of appropriate stabilizer and concentration can help reduce the size of the particles, narrow distribution and prevent nanoparticles from agglomeration resulting in a more stable nanoparticles preparation (Van Eerdenbrugh *et al.,* 2008). There are several reports highlighting the important role of matrix forming substances such as sucrose, mannitol before solidification processes such as freeze drying and spray drying to overcome

the instability issues (Abdelwahed *et al.,* 2006 ; Kesisoglou *et al.,* 2007 ; Van Eerdenbrugh *et al.,* 2008).

Curcuminoids are known to degrade on exposure to light, on acidic and alkaline hydrolysis and oxidation (Pfeiffer *et al.,* 2003; Wang *et al.,* 1997). The chemical stability of curcuminoid in SLN when incorporated into a model cream was significantly improved (Tiyaboonchai *et al.,* 2007). Very recently, our group reported a preliminary study on the stability of lab-scale prepration of curcumin encapsulated nanoparticles at 25°C and 60% RH (refrigerated test conditions) for 3 months (Shaikh *et al.,* 2009) and seem to be stable though the duration of exposure is short and should be at least 6 months to qualify for regulatory requirements.

2.1.3 Effect of homogenization time

According to Stroke's law, the surface free energy of the droplets present in the internal phase in an emulsion is inversely proportional to the diameter (Leroux *et al*., 1995). To decrease the surface energy, the stabiliser molecules align themselves at the interface thus decreasing the globule size further. Homogenization is a key variable in emulsion processes that has a significant influence on the size and distribution of nanoparticles during their preparation and can be achieved by ultrasonic, pressure and mechanical means. Small sized bench-top pressure homogeniser (Polytron PT4000; Polytron Kinematica, Switzerland, the diameter of the rotor was 9mm and the external diameter of static shaft was 11.8 mm) suitable for working volumes of 0.1-250 ml was used for this project and the effect of homogenization time (5 min, 15 min and 30 min) on nanoparticle characteristics was investigated.

2.1.4 Centrifugation

Centrifugation is an essential step in emulsion based nanoparticles preparation techniques that allows separation of excess/free surfactants or un-entrapped drugs from the final preparation (Sahoo *et al.,* 2002; Italia *et al.,* 2011). The excess free surfactant if not removed can compromise absorption/cell uptake as well as cause toxicity. The free drug can also interfere in further processing conditions such as freeze drying. Centrifugation is followed by washing nanoparticles with water or water and methanol mixture to remove free surfactant and drugs. However, the centrifugation force can be very critical where very high force cause caking and difficulties in re-suspending of nanoparticles while low force can result in significant loss of nanoparticles thereby low yield of nanoparticles (Italia *et al.,* 2011).

2.1.5 Freeze drying

As discussed in section 2.1.2, nanoparticles when in aqueous suspension, the polymer undergoes slow degradation and leakage of drug. Hence water has to be removed to preserve the physical and chemical properties and achieve stability of nanoparticles (Abdelwahed *et al.,* 2006). Freeze drying also known as lyophilization is the most accepted process used to convert a solution or suspension to solid form in the pharmaceutical field by sublimation and desorption under vacuum (Franks, 1998). To avoid the stress of extreme drying and freezing of nanoparticles during this process, protectants usually sugars such as monosaccharides and disaccharides are used. The resulting freeze dried product can be reconstituted as and when required.

Freeze drying is a complex process consisting of three cycles. The product is first frozen or solidified followed by primary drying (ice sublimation) and secondary drying during which the frozen water is desorpted. In addition to the concentration of polymer, surfactant and cryoprotectants used, the pressure, temperature and the duration of each stage of the process also plays an important role in the success of the final freeze dried products (Abdelwahed *et al.,* 2006).

The current study is focussed on the optimization of a preparation process for pilot scale batches and to test the shelf-stability of the formulations under room as well as refrigerated test conditions for storage to fulfil regulatory requirements. The objective will be achieved by the following specific aims:

2.2 Specific aims

- To investigate the role of homogenization time on particle characteristics.
- To optimize the freeze drying process resulting in product that retains the particle characteristics of fresh preparations.
- To test the integrity of the nCUR upon storage at room and refrigerated test conditions.

2.3 Materials and methods

2.3.1 Materials

Poly (lactide-co-glycolic) acid (PLGA) (Resomer R503H; MW 35–40 kDa) was purchased from Boehringer Ingelheim, (Ingelheim, Germany). Polyvinyl alcohol (PVA) (MW 30–70 kDa), sucrose and ethyl acetate were purchased

from Sigma-Aldrich (Poole, UK). Curcumin was purchased from Indsaff, Punjab, India. HPLC grade acetic acid, acetone, acetonitrile, methanol were obtained from Fisher Scientific UK Limited. Distilled water was prepared in-house.

2.3.2 Preparation of nanoparticles

Modified emulsion-diffusion-evaporation method which was previously developed (Devadasu *et al.* 2008) was used to prepare the nanoparticles. The polymer, PLGA (50mg) and curcumin (7.5mg) were dissolved in 2.5 ml of ethyl acetate and allowed to stir at 1000 rpm for 1 h obtaining a homogeneous solution. During this time, PVA (50 mg) used as the stabilizer was dissolved in distilled water (5 ml). The organic phase containing the drug and polymer was then added drop by drop to the stabilizer solution while stirring and was further stirred for 5 min. Later, the emulsion was homogenized at 15,000 rpm for 5 min and transferred to 25 ml water to further promote diffusion and was stirred overnight to confirm complete evaporation of the organic solvent. The nanoparticles suspension was then centrifuged (Section 2.3.4) to separate any unbound stabilizer and free curcumin in the preparation. The scheme of particle preparation is depicted in Fig. 2.1.

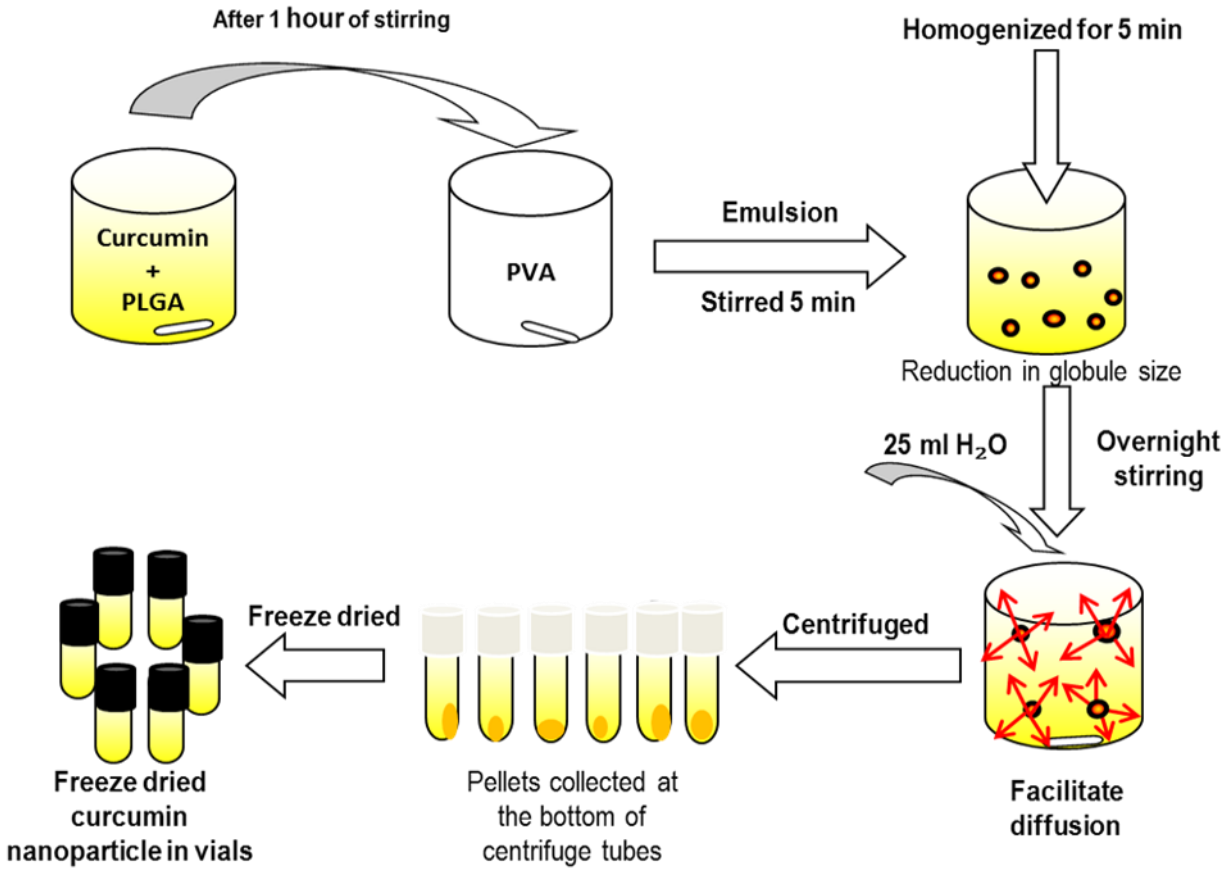

Figure 2.1 A schematic representation of particle preparation methodology.

2.3.3 Centrifugation

For the removal of un-entrapped curcumin and PVA the suspension obtained after overnight stirring was centrifuged (3K30, Sigma, USA or Avanti® J-E centrifuge, Beckman Coulter Inc., Fullerton, CA, USA). The suspension was centrifuged at 14,000g for 30 min and the supernatant discarded. The pellet was re-dispersed in 1 or 2 ml of distilled water as required. Characterization was carried out for particle size, zeta potential and entrapment efficiency and was further processed for freeze drying.

2.3.4 Freeze drying

5% sucrose (w/v) was added as a lyoprotectant to the pellets collected after centrifugation (Shaikh *et al.,* 2009). The suspension was then briefly vortexed until sucrose dissolved and divided into 2 ml aliquots into the glass vials (5 ml).

A bench top freeze-drier (EPSILON 2- 4, Martin Christ, Germany) using optimized stepwise freeze drying cycle as mentioned in Table 2.1 was adapted and the freeze dried nanoparticles were easily re-suspended in 1 ml of distilled water and used for characterization and further studies.

Table 1.1 Conditions for freeze drying

Conditions	Shelf temperature[°C]	Vacuum [mBar]	Safety pressure
Freezing (8 h)	-50	-	1.650
Main drying (48 h)	-50	0.0035	1.650
Final drying (12 h)	+20	0.0035	1.650

The freeze drying has also been simplified which was thoroughly optimised, were the nanoparticles were pre-freezed at -80°C for 12 h and then placed on the tray of MicroModulyo (Thermo Savant) and dried for 48 h at -50°C under vacuum 0.0035 mBar. The freeze dried products were easily re-suspended in 1 ml of distilled water and used for characterization and further studies.

2.3.5 Scale-up of curcumin encapsulated nanoparticles

The idea was to simulate production on intermediate scales before larger scale work was undertaken. In this dissertation, a 10-fold (500 mg polymer) increase in the batch size was investigated and the characteristics were compared to that of lab-scale preparation 50 mg. From the parameters involved in the preparation the homogenisation step is crucial for scale-up; therefore, we investigated the duration of homogenisation on particle characteristics keeping the rest of the parameters similar including the homogenization speed. Scale-up production of nCUR was similar to that of

the standard procedure as mentioned above in section 2.3.2 with exceptions in homogenization duration and the material quantities used. In brief the procedure is as follows: PLGA (500 mg) and curcumin (75 mg) were dissolved in ethyl acetate (25 ml) (under stirring over a period of 1h. PVA (1%w/v) was dissolved in water (50 ml) under stirring for 1h. The PLGA+curcumin solution (organic phase) was emulsified into PVA solution (aqueous phase) over 30 min under stirring at 1,000 rpm. The emulsion was homogenised (Polytron PT4000; Polytron Kinematica, Switzerland) at 15,000 rpm using for 5 or 15 or 30 min. The emulsion was then transferred to water (250 ml) and stirred (1,000 rpm) overnight to ensure complete evaporation of ethyl acetate. The curcumin encapsulated nanoparticles suspension was centrifuged at 14,000g for 30 min and the pellet was collected. All the preparations have been performed in triplicates.

2.3.6 Characterization of nanoparticles

2.3.6.1 Particle size distribution and Zeta potential

The characterization of the nanoparticles for size (average of 5 measurements for one batch) and zeta potential (average of 20 measurements for a batch) was performed using a zetasizer (Nano ZS, Malvern Instruments, Malvern, UK). This technique measures the diffusion of particles moving under Brownian motion where the laser illuminates the particles and the intensity fluctuations is analysed in the scattered light converting this to size and a size distribution (polydispersity index PDI, different size population in same sample) on the basis of Stokes-Einstein relationship.

The zeta potential is the charge of the particles which helps to determine the particle stability in dispersion. It was estimated under an electric field on the basis of electrophoretic mobility.

2.3.6.2 Atomic Force Microscopy (AFM)

The topography of the nanoparticles was analysed using AFM. 5 µL of the particle suspension (50µl of the pellet further diluted) was deposited onto a freshly cleaved mica surface (G250-2 Mica sheets 1" x 1" x 0.006"; Agar Scientific Ltd, Essex, UK), and air dried for 30 min before AFM imaging. The Bruker MultiMode with NanoScope IIID Controller Scanning Probe Microscope (Digital Instruments, Santa Barbara, CA, USA; Bruker Nanoscope software Version 6.14r1) was used to procure topographic images by tapping mode at a frequency of 73 kHz under ambient conditions by scanning mica surface in air. The resolution of the images was 512 by 512 pixels. Sharp silicon probes were used to obtain AFM measurements (FESP; nominal length (l_{nom}) = 225 µm, width (w_{nom}) = 28 µm, tip radius (R_{nom}) = 8 nm, resonant frequency (υ_{nom}) = 75 kHz, spring constant (k_{nom}) = 3 N m^{-1}; Bruker Instruments SAS, Dourdan, France). The other scanning parameters were as follows: scanning speed 1.0 – 1.5 Hz, integral and proportional gains 0.3 and 0.5, respectively and points set at 0.5 – 0.8 V. Atleast five random spot surface areas in two different samples were scanned.

2.3.7 Entrapment efficiency

Percentage entrapment efficiency was calculated as the ratio of amount of curcumin entrapped in nanoparticles to the amount of curcumin used in preparation of nanoparticle using equation below:

$$\%\ curcumin\ entrapment\ efficiency = \frac{amount\ of\ curcumin\ entraped\ in\ NP}{amount\ of\ curcumin\ used\ for\ formulation} \times 100$$

2.3.7.1 Adaption and validation of analytical method

HPLC method previously developed in our laboratory was adapted and validated with slight modifications (Shaikh *et al.,* 2009). The stock solution of curcumin was prepared by dissolving 10 mg of curcumin in 100 ml methanol in volumetric flask. The mixture was sonicated thoroughly and standard solutions of curcumin with concentrations of 1, 2, 5, 10, 15 and 20 μg/ml were prepared using methanol.

In vitro analysis of curcumin was performed using Thermo HPLC system (Thermo, USA) made up of surveyor auto sampler, surveyor LC pump and Photodiode Array (PDA) detector with gradient elution on 5 μm column Thermo ODS 100RP-18 (150 x 4.6mm) end-capped using mobile phase composed of Methanol: acetonitrile: acetic acid at a flow rate of 1ml/min Table 2.2. The injection volume was 50 μl and retention time of curcumin was ~ 6 min. The detection wavelength (λmax) for curcumin was 425 nm. All samples were analysed in triplicates.

Table 2.22 Mobile phase gradient for *in vitro* curcumin analysis

Time (min)	Acetonitrile (%)	Methanol (%)	2% Acetic acid (%)
0	20	20	60
3	50	50	00
5	50	50	00
9	20	20	60

2.3.7.2 In vivo HPLC method

The *in vivo* analysis of curcumin was performed using a method previously developed in our laboratory with slight modifications (Shaikh *et al.,* 2009). Fresh blood collected in heparinised EDTA tubes from Sprague Dawley (SD) rats was centrifuged at 3000 rpm for 10 min at 3°C to separate plasma from RBCs. The supernatant was carefully collected and stored in refrigerator at 4°C. A stock solution of curcumin of concentration 10µg/ml was prepared by weighing 10 mg curcumin and dissolving in 10 ml of HPLC grade methanol. Further dilutions were prepared to make stock solutions of concentration 10µg/ml and 1µg/ml and used for developing *in vivo* calibration curve. 250µl of plasma was taken in a 2 ml eppendorf tube. To the plasma, stock solution of curcumin (to avoid error equal quantities were made up using methanol). As curcumin is stable in acidic condition 25 µl of 2.8% acetic acid was added and 50 µl of internal standard (100µg/ml) was added. 17 β –estradiol acetate dissolved in methanol was used as an internal standard and vortexed for 20 sec. Extraction was carried out by adding 1.2 ml of ethyl acetate. This was vortexed again for 15 min and centrifuged at 10000 rcf for 10 min at 4°C to separate plasma proteins. The supernatant was collected carefully using a

micropipette in eppendorf tube and let to dry overnight under fume hood. To the residue, 130 µL of methanol was added and transferred into HPLC inserts. Standard solutions of 10, 20, 40, 80, 120 and 200ng/ml were prepared in triplicate by above mentioned method. The flow rate was 1.0ml/min in gradient mode using 2.8% acetic acid, methanol and acetonitrile, in concentration as shown in Table 2.3. 17β-estradiol acetate and curcumin was analysed at 280 nm and 425 nm respectively. All samples were analysed in triplicates.

Table 2.3 Mobile phase in gradient elution

Mobile phase	Time	A%	B%	C%	Curve
A-MeCN	**0**	35	55	10	
B-2.8% Acetic	**4**	45	25	30	3
acid	**6**	40	30	30	3
C- MeOH	**8**	35	55	10	3

2.3.8 ICH stability study

The nanoparticles formulations were subjected to accelerated ICH stability test conditions for room temperature as well as refrigerated storage. The freeze dried vials were sealed and placed in the stability chamber at room temperature storage conditions 40°C ± 2°C/75% RH ± 5% RH (LEEC cooled humidity cabinet, ultrasonic type, Model: SFC3C/RH) and storage in a refrigerator conditions 25°C ± 2°C/ 60% RH ± 5% RH (MMM Climacell cooled humidity cabinet, Model: 404L incubator) for six months. All sampling were done in triplicates. Physical appearance was noted each month. Characterization for size and topography, entrapment efficiency were

performed at a frequency of 1 month for six months and the results were compared against fresh samples.

2.3.8.1 X-ray diffraction (XRD) analysis

Further we have confirmed the crystalline nature of curcumin over a period of time using XRD techniques. The XRD patterns of native curcumin, sucrose, nCUR and six month stability samples were achieved using the X-ray diffractometer (Bruker axs, D8 advance) with source of radiation by Cu. Measurements were performed at a voltage of 40 kV and 25mA. The scanned angle was set from $2^\circ \leq 3 \geq 50^\circ$ and the scan rate was 3° min^{-1}.

2.4 Results and discussion

2.4.1 Preparation and characterization of nCUR

nCUR were prepared successfully reproduced by previously developed emulsion-diffusion-evaporation technique (Shaikh *et al*., 2009). The initial loading of curcumin was limited to 15% w/v of the polymer due to solubility of curcumin in ethyl acetate and drug holding capacity of the PLGA used in this study. Increased drug loading resulted in larger size nanoparticles. The Z-average particle size of nCUR was 282.50 ± 5.72 nm (Fig 2.2) and PDI of 0.14 ± 0.06 at 15% w/w drug loading. The PDI value close to 0 indicated highly mono-disperse nature of particles The prepared nCUR under AFM revealed spherical shape and had smooth surface (Fig 2.3) and the size is similar to the data obtained by dynamic light scattering.

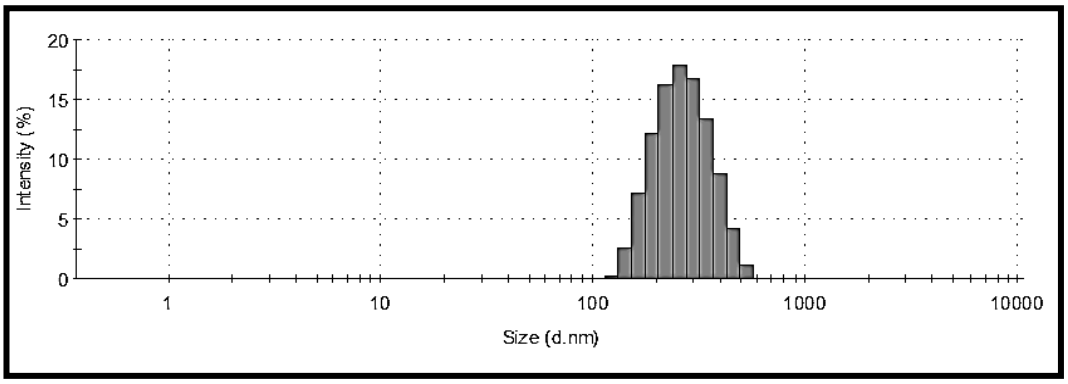

Figure 2.2 Representative size distribution by intensity plot for nCUR.

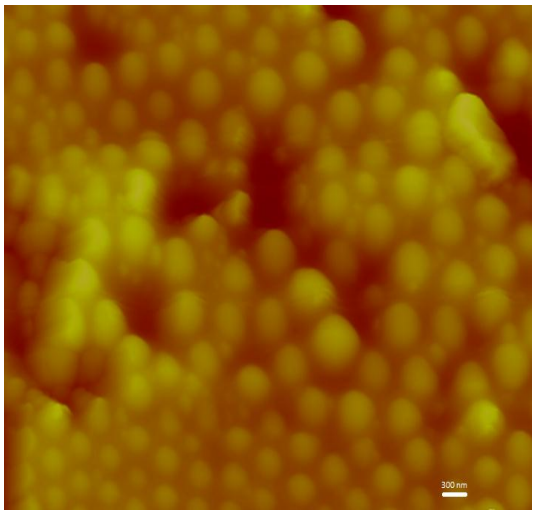

Figure 2.3 AFM photomicrograph of nCUR.

2.4.2 Effect of homogenisation time on nCUR characteristics

In case of nanoparticles preparation homogenization of the emulsion seems to play a major role in controlling the particle size as well as poly-dispersity. Keeping the rest of parameters constant and changing just one factor, the homogenisation time, it was noted that the particle size and PDI decreased with increase in the homogenisation duration and were uniformly distributed (Table 2.4). The batch to batch variation was insignificant when the emulsion was homogenized for 30 min in comparison to 5 and 15 min.

The lab scale batches were compared for particle size and entrapment efficiency against 5 and 10 fold increases in batch sizes. AFM images (Fig 2.4) represented the spherical shaped nanoparticles and measured average particles size was comparably same with particle size measured by DLS method.

Table 2.2 Effect of homogenisation time on particle characteristics of NPs

Homogenisation time (min)	Mean particle size(nm) ±sd	Polydispersity index (PDI)
5	431.76±51.63	0.25±0.06
15	380.5 ±7.70	0.28±0.09
30	288.73±3.44	0.15±0.01

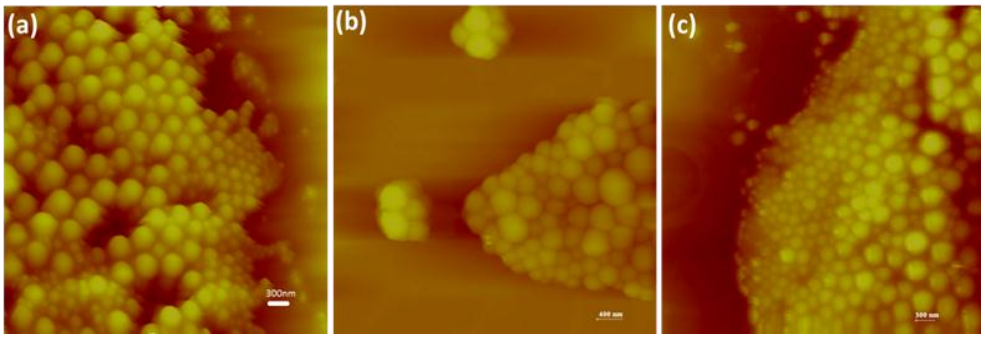

Figure 2.4 AFM photomicrographs of nCUR at different homogenisation durations (a) 5 min (b) 15 min and (c) 30 min.

2.4.3 Scale- up of nCUR

It is a widely accepted fact that the formulations prepared in a laboratory environment often face significant challenges in production to large quantities, therefore it is important to move in an incremental fashion from lab to pilot before taking it to commercial scale. This is due to the fact that the instruments intended for the laboratory setting differ from the ones required

for large scale production. In case of nanoparticles preparation homogenization of the emulsion seems to play a major role in controlling the particle size as well as PDI. The conventional homogenizer in this case seems to be good for moving from lab to pilot scale; however this may not work at commercial level but good enough for chronic studies and preclinical toxicity assessment.

The feasibility of scale-up preparation of nCUR has been successfully demonstrated where homogenisation plays a pivotal role in the scale-up. During homogenisation, the globule size is reduced but the surface area of any two globules is increased and is inversely proportional and this is where the stabilisers play their part. By aligning themselves in the interface of the two phases the stabilisers reduce the surface tension hence reducing the thermodynamic instability.

We noted the homogenisation duration of 30 min at 15000 rpm resulted in attaining an acceptable particle size when the batch size was increased by 5 and 10 fold, while homogenising 5 min was sufficient for lab scale batch (50 mg). The entrapment efficiency obtained from the lab-scale was compared to the pilot scale resulting in statistically insignificant variation ($p>0.05$) in particles size and entrapment efficiency. The particle characteristics of the nCUR obtained at lab scale and pilot scale are shown in Table 2.5.

Table 2.3 Effect of batch size on particle characteristics of nCUR

Batch size	Mean particle size(nm) ±sd	Polydispersity index PDI	Entrapment efficiency (%) ±sd
Lab scale	282.50 ± 5.72	0.14 ± 0.06	53.33±4.52
5–folds	296.63±5.72	0.12±0.06	57.42±4.11
10-folds	288.8±3.54	0.15±0.02	52.46±4.33

2.4.4 Freeze drying of nCUR

It is known that the freeze drying process can result in aggregation, affect the surface morphology, and the internal structure of the nanoparticles, to some extent lyoprotectants/cryoprotectants can avoid the damage caused during this process. The use of sucrose as a cryoprotectant for nCUR was previously reported by our group (Shaikh *et al.*, 2009). Though the previous process led to easy to re-suspend intact fluffy cake with aesthetic appearance, it led to increase in the particle size therefore we modified the process as mentioned in section 2.3.4.

The nCUR (2 ml) were successfully freeze dried using sucrose (5% w/v) as a cryo/lyo-protectant resulting in intact fluffy cake. The freeze drying process has not introduced any significant changes in the particle size or entrapment efficiency when compared to those of fresh samples. The freeze dried nCUR was found to be re-suspended easily in water by vortexing for 30 sec. The AFM image further confirms that the particles were spherical in shape with smooth surfaces after freeze drying process. These results once again suggest freeze drying can be effectively used to preserve the nanoparticles for longer

duration without having their properties changed from those of freshly prepared particles.

2.4.5 Adaption and validation of analytical method

2.4.5.1 HPLC for in vivo estimation of curcumin

The concentration of curcumin used for generation of calibration curve was in the range of 10 to 200 ng/ml with linearity of correlation coefficient, r^2, value of 0.997. The curcumin was eluted at RT of 6 min and IS at 5 min (Fig 2.5).

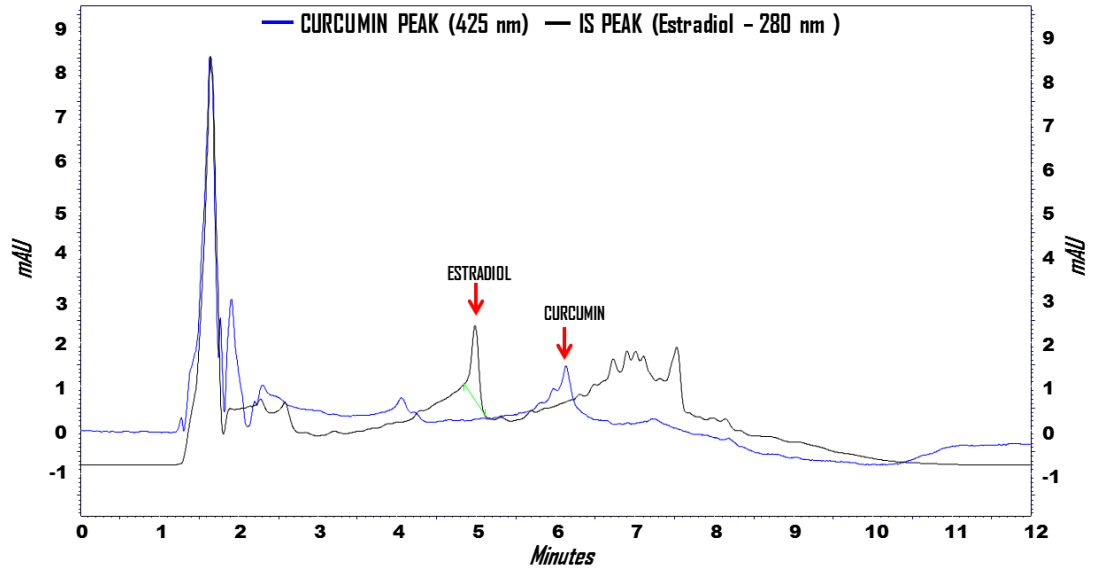

Figure 2.5 Representative chromatogram of plasma sample spiked with curcumin and IS at 425nm and 280 nm respectively.

2.4.6 ICH stability study

Literature lacks, if not very little is available on regulatory shelf-stability testing of most investigated PLGA nanoparticles in general and particularly

those of nCUR which is picking up pace considering the nanoparticles ability to improve *peroral* bioavailability of this very high dose natural anti-inflammatory compound. A majority of the pharmacological interventions with curcumin need repeated dosing, rather in very high doses (Kumar *et al.,* 2005; Lin *et al.,* 2007), though nanoparticles is not a preferred approach for high dose compounds (certain degree of risk associated with this statement, but true to large extent) by the rule of thumb (Devadasu *et al.,* 2013), however, academic research is still on with a hope that nanoparticles will lead to dose reduction (Shaikh *et al.,* 2009; Devadasu *et al.,* 2011; 2012; Dandekar *et al.,* 2010; Pawar *et al.,* 2012). Under these circumstances it is very important that the shelf-life is assessed so that formulation is made and stocked for chronic preclinical studies. Occasionally there are studies documented in the literature claiming stability of the curcumin formulations proven, however the studies are not conducted according to regulatory requirements (Ranjan *et al.,* 2012). It is also well known that PLGA is sensitive to moisture and temperature and undergoes degradation by simple ester hydrolysis as well as amorphous form of the drugs in PLGA nanoparticles tend to recrystallize on storage at high temperature and humidity conditions. In the current study, nCUR were stable at room temperature as well as refrigerated storage conditions without shrinkage and collapse at the end of six months (Figs 2.6a and 2.6b).

Figure 2.6 Photograph of nCUR subjected to ICH stability test conditions (a) fresh and 1-6 months at room temperature conditions (b) fresh and 1-6 months at refrigerated conditions**.**

From the AFM analysis the size of nanoparticles, shape and surface properties are unaltered when compared to those of freshly prepared samples stored at both conditions (Figs 2.7 and 2.8).

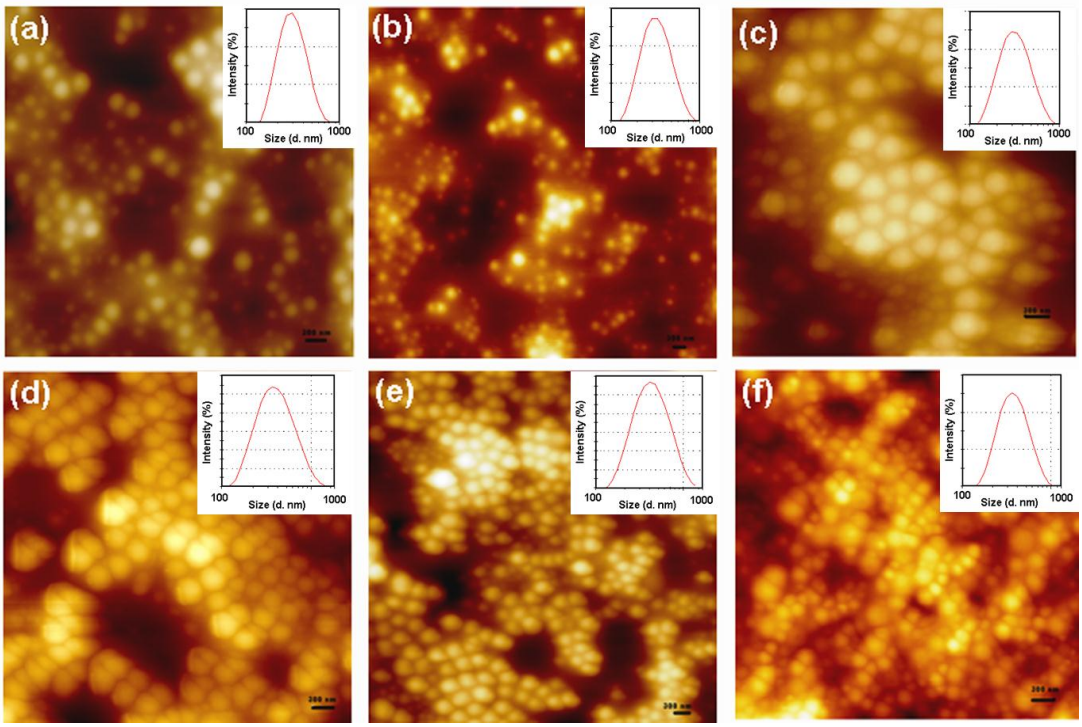

Figure 2.7 AFM photomicrographs and insert particle size distribution plot of (a) 1 (b) 2 (c) 3 (d) 4 (e) 5 and (f) 6 month long term stability samples (40°C ± 2°C/75% RH ± 5% RH) (10 μm x 10 μm, with a 300 nm scale bar).

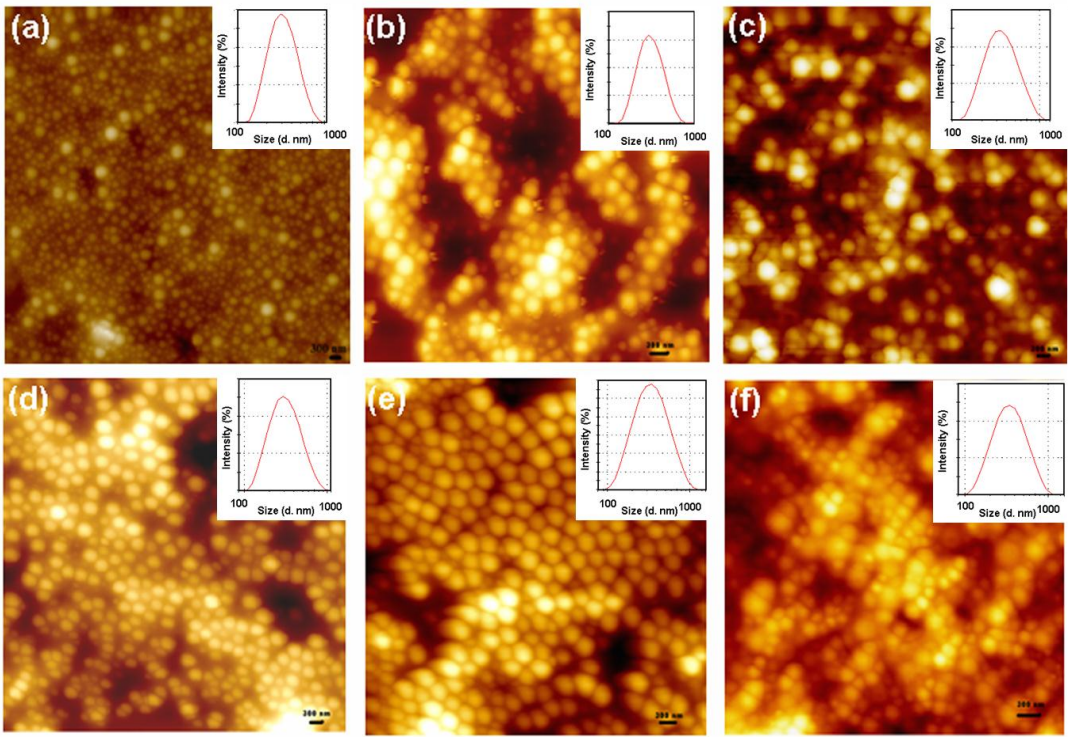

Figure 2.8 AFM photomicrograph and insert particle size distribution plot of (a) 1 (b) 2 (c) 3 (d) 4 (e) 5 and (f) 6 month long term stability samples (25°C ± 2°C/ 60% RH ± 5% RH) (10 μm x 10 μm, with a 300 nm scale bar).

No significant change in the particles characteristics and the content of curcumin (Figs 2.9, Fig 2.10, Fig 2.11) was observed. The physical appearance remained unchanged and reconstitution with distilled water was easy.

The data was analysed using multi factor ANOVA. $p > 0.05$ is considered insignificant.

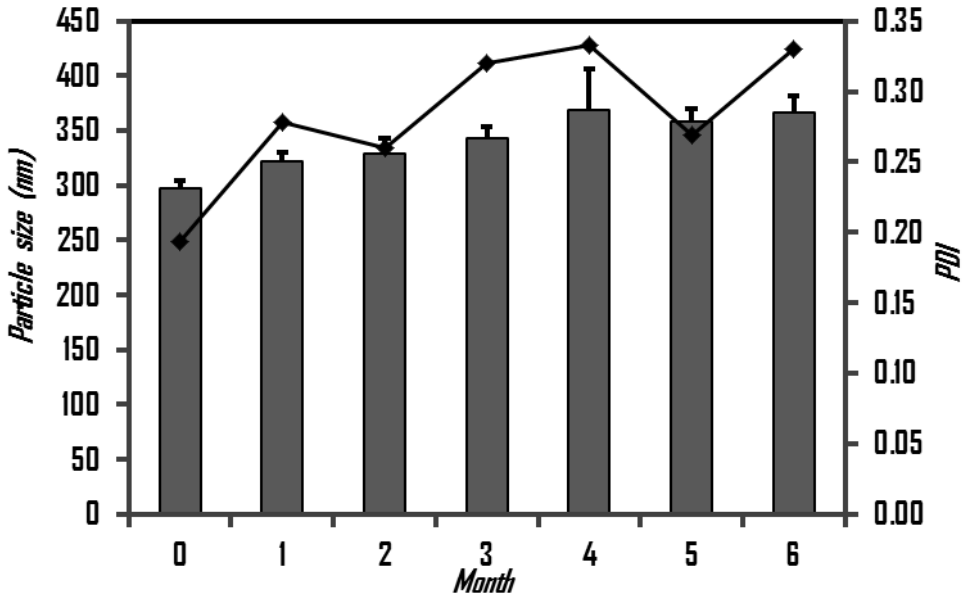

Figure 2.9 The average particle size and PDI of fresh nCUR (freeze dried and reconstituted) compared to that obtained at different time intervals during room temperature storage (40°C ± 2°C/75% RH ± 5% RH).

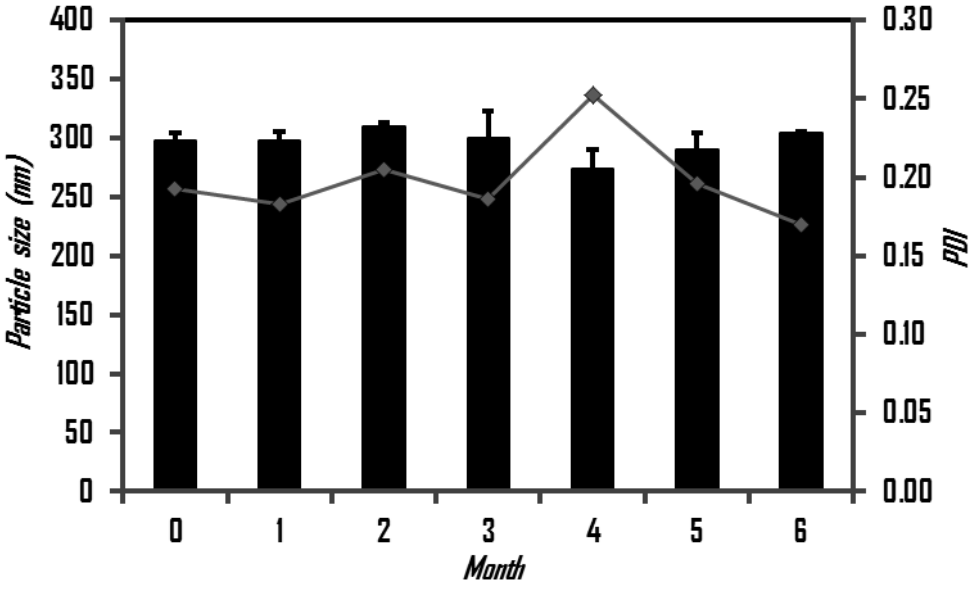

Figure 2.10 The average particle size and PDI of fresh nCUR (freeze dried and reconstituted) compared to that obtained at different time intervals during refrigerated storage conditions (25°C ± 2°C/ 60% RH ± 5% RH).

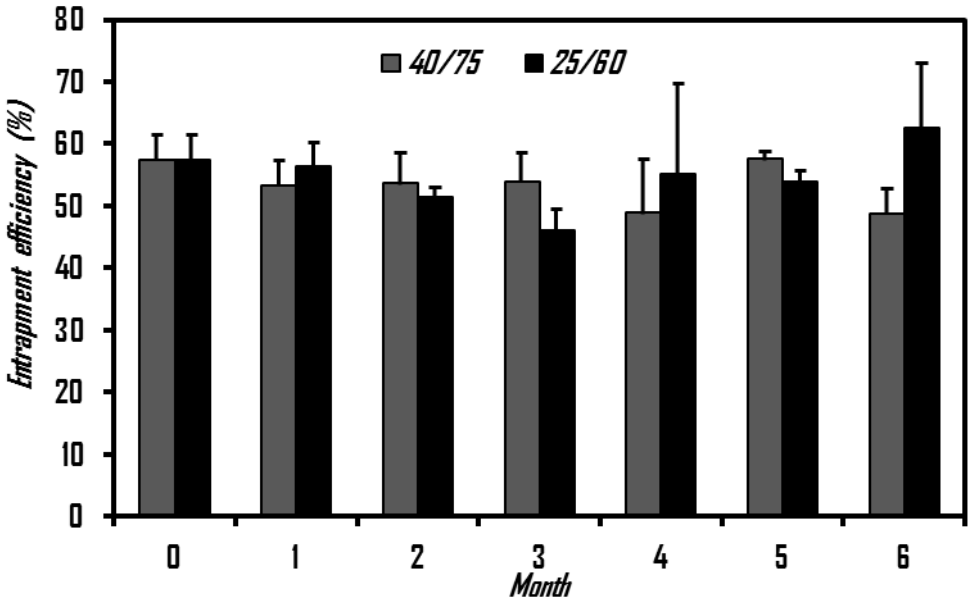

Figure 2.11 Percentage entrapment efficiency of fresh nCUR (freeze dried and reconstituted) compared to that obtained at different time intervals during stability test conditions**.**

The XRD analysis of freeze dried nCUR (Fig 2.12c) and those subjected to room temperature (40°C ± 2°C/75% RH ± 5% RH) (Fig 2.12d) and refrigerated storage conditions (25°C ± 2°C/ 60% RH ± 5% RH) (Fig 2.12e) did not show any characteristic peaks and stayed amorphous at the end of six months. The peaks seen in the sucrose and native curcumin (Fig 2.12a, 2.12b) are characteristic and can be inferred due to its crystalline structure. During preparation of nCUR, curcumin is converted to amorphous state due to intermolecular interaction occurring in the PLGA matrix (Abdelwahed *et al.,* 2006).

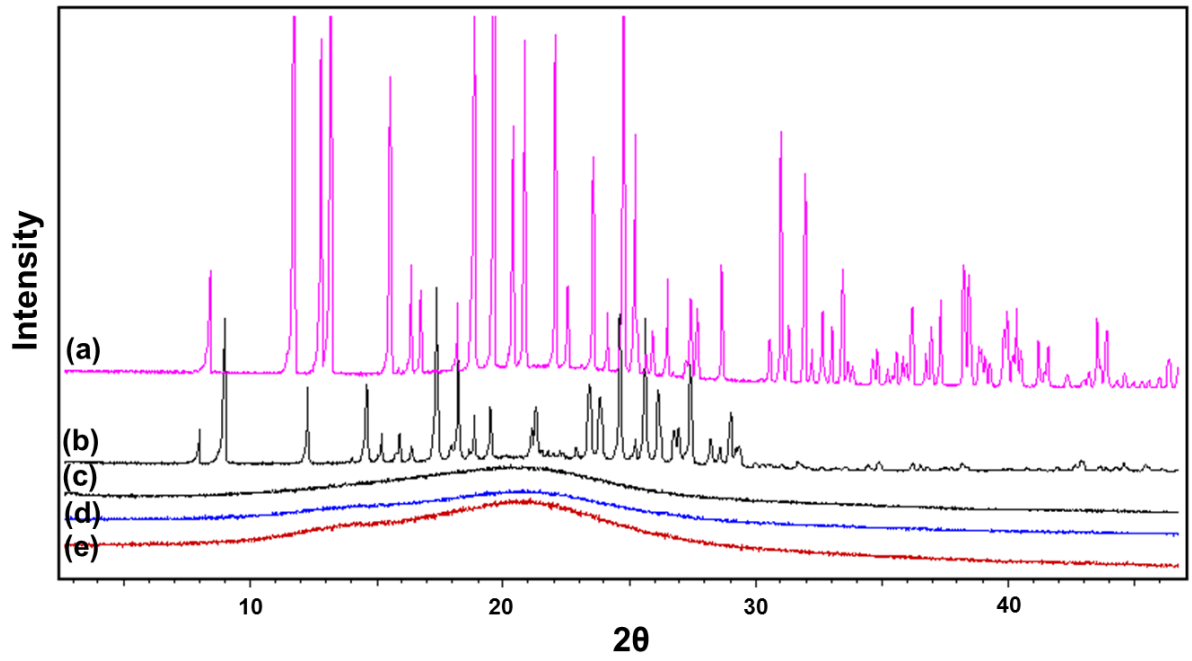

Figure 2.12 XRD spectra of (a) sucrose, (b) curcumin, (c) nCUR at time 0 (d) nCUR subjected to room temperature test conditions at 6 months and (e) nCUR subjected to refrigerated test conditions at 6 months.

2.5 Conclusion

- The feasibility of scale-up of nCUR was successfully tested at 10 times larger batch sizes compared to lab-scale.
- The homogenisation duration is found to be influential parameter in the scale-up (500 mg) wherein 30 min at 15000 rpm led to particles with comparable characteristics of lab-scale (50 mg) preparations homogenised at 15000 rpm for 5 min.
- The freeze drying process adapted in the current study does not affect the nCUR properties and are comparable to fresh preparations.
- The nCUR retained the characteristics such as size, PDI, surface morphology, re-suspendability, amorphous state and entrapment efficiency during and after accelerated stability testing both at room temperature as well as refrigerated conditions.

3 EVALUATION OF nCUR IN RAT MODEL OF DIABETIC CATARACT

3.1 Introduction

In the 21st century, DM has become the most common and challenging metabolic disorder with T2D making up to 85 to 95% of the two types (Melmed *et al.,* 2012). About 552 million people worldwide have been estimated to have diabetes before 2030 (IDF, Diabetes Atlas, 5th Edition). Diabetes by itself and its complications is the fourth or fifth major cause of death in most of the countries (IDF Diabetes Atlas, 5th Edition). Diabetes can be inherited or acquired, caused due to insufficient insulin functionality causing hyperglycemia and development of micro- and macro-vascular complications (Fowler 2008). DM belongs to the chronic autoimmune diseases associated with increased production of free radicals. Several non-enzymatic, enzymatic and mitochondrial pathways can be considered as likely candidates for formation of oxygen free radicals in cells because of hyperglycaemia contributing to the development and progression of diabetes and treatment with antioxidants might be an effective strategy for reducing diabetic complications. Antioxidant treatment can exert beneficial effects in diabetes by preservation of *in vivo* beta-cell function, reduction of mitochondrial mutations, elevation of antioxidant enzyme levels, and prevention of lipid peroxidation thereby reducing hyperglyceamia and oxidative stress which are considered to be the main culprits in the development and progression of diabetic complications (Giugliano *et. al.,* 1995).

Diabetes is considered as the leading cause of new blindness and end stage renal failure due to pathological effect on microvasculature (Alberti &

Zimmet, 1998; Nathan, 1993). Macrovascular complications of diabetes result in coronary artery disease, cerebrovascular disease and peripheral vascular disease which are among the leading cause of death in the diabetic patients (www.idf.org). Overall life expectancy for patients with diabetes is ~7-10 years shorter than for people without diabetes. The Diabetes Control and Complications trial (DCCT) demonstrated that tight control of glucose levels in blood is effective in managing DM. However it was observed even optimal controlling of the levels in blood glucose could not halt the diabetic complications proposing additional treatment strategies are essential (Shamoon *et al.,* 1993). The role of strict control of blood glucose in preventing micro-vascular complications (eg, retinopathy, neuropathy and nephropathy) is being explained as effective by the United Kingdom Prospective Diabetes Study (UKPDS 33) whereas its role in preventing macro-vascular (eg, coronary heart disease and stroke) complications remains elusive (Gerstein *et al.,* 2008, 2012; Patel *et. al.,* 2008; Ferguson & Sattar 2012). A prospective observational study carried out by the UKPDS concluded reduction in glycated haemoglobin (HbA1c) reduces the risk of complication (Turner *et al.,* 1998; Stratton *et al.,* 2000). The cumulative incidence of long term complications showed lower incidences of diabetic nephropathy, proliferative retinopathy and cardiovascular diseases than what was reported previously on intensive treatment in T1D (Nathan *et al.,* 2009).

3.1.1 Diabetic cataract

The major cause of visual impairment in diabetic patients is cataract which remains a continuing and greatest challenge. The literature evidence suggests

the incidence and progression of cataract increases in diabetic patients (Harding *et al*., 1993; Kahn *et al*, 1977) and are at more risk of developing a cataract at an earlier age (Klein *et al*., 1995; Ederer *et al*., 1981). The overall outcome of cataract surgery in diabetic patients is poor compared to the non diabetic patients. There is increased risk of post-operative inflammation, acceleration of retinopathy and macular changes in diabetic patients post operatively (Pande *et al*., 1996). Although the aetiology of cataract is not fully understood, oxidative damage is considered as a major mechanism in the development of cataract (Spector, 2000). The major source of oxidative stress in the lens was due to the polyol pathway causing AR induced osmotic stress due to accumulation of sorbitol (Chung *et al*., 2003). The delay of progression and maturation of cataract in rats supplemented with curcumin was reported although it did not have influence on hyperglycemia (Suryanarayan *et al.,* 2003, 2005; Kumar *et al*., 2005; Muthenna *et al*., 2009).

A diabetic research study conducted on the younger generation aged 12-40 years (ASDIAB Study), reported the prevalence of DR was least among Indians (5.3%) whereas the prevalence was 10% and 15.1% in Malays and Chinese respectively (Rema & Mohan, 2002) and lower in south India compared to the other populations and may be attributed to the type of diet rich in carbohydrates and use of anti-inflammatory agents such as curcumin (Rema & Pradeepa, 2007). A recent case report suggests consumption of curry diet has health benefits (Seshi, 2012).

Lack of curcumin oral bioavailability is most commonly believed to pose significant challenges in the development of curcumin based medicines (Kumar 2012). Recently, our group reported a 9-fold increase in oral bioavailability of nCUR compared to that of plain curcumin in rodents (Shaikh *et al*., 2009). Building on our previous data the major objective of this

study was to investigate the efficacy of nCUR in preventing/delaying diabetic cataract in rats.

3.1.2 Diabetic cataract model

The animal models of DM can be either spontaneous or secondary. In the secondary models diabetes is achieved by removal of pancreas, by drug treatment of by biomolecular means. In 1880s, pancreatomised models (dogs and rabbits) were used (Rees & Alcolado 2005) which was commonly used in larger animal group and about 90-95% removal of pancreas was a must to induce diabetes (Akbarzadeh *et al.,* 2007). But today most of the experiments are carried out in rodents and to necessitate and mimic the diabetic pathology in humans, drug induced models are being used commonly. In early 1940s, alloxan was first used for induction of diabetes by necrosis of the endocrine beta cells (Dunn & McLetchie, 1943). Later on in the 1960s, STZ was developed which was used to treat metastatic cancer of pancreatic islet cells and now has been used as a popular choice for inducing diabetes in animal models (Weiss, 1982).

Several mechanisms by which STZ induces diabetes have been explained (Zahner & Malaisse, 1990; Wang & Gleichmann, 1998). The dose for inducing diabetes depends on the strain, animal species and route of administration (Hayashi *et al.,* 2006). This agent is widely used as diabetogenic compound as it mimics the pathogenesis in the generation of diabetes as in humans where the primary phenomenon is due to autoimmune response (Notkins & Lernmark, 2001), has relatively longer half-life, sustained hyperglycemia for longer duration, development of complications with lesser incidence of ketosis and mortality (Ozturk *et al.,* 1996).

Rodents upon administration of STZ results in hyperglycemia and left untreated over a period of time will develop micro- and macro-vascular diabetic complications by altering the levels of pro-inflammatory and inflammatory biomarkers (Brownlee, 2001; Obrosova *et al.*, 2005a,b). Hence, we used streptozotocin induced DM model to study the ability of nCUR in managing diabetic cataract.

3.2 Specific aims

- To evaluate the potential of nCUR in delaying diabetic cataract in rat model.
- To prove nCUR works on similar molecular mechanisms responsible for cataractogenesis in diabetes as CUR, but more efficacious.

3.3 Materials and methods

3.3.1 Materials

Streptozotocin (STZ), NADPH, NAD, 2-thiobarbituric acid, DL-glyceraldehyde, 1,1,3,3-tetraethoxy propane (TEP), lithium sulphate , β-mercaptoethanol, sorbitol, sorbitol dehydrogenase, BSA, 2,4-dinitrophenylhydrazine, pyrogallol, diethylenetriaminepentaacetic acid and EDTA were purchased from Sigma-Aldrich (St. Louis, MO).

3.3.2 Preparation and characterization of nCUR

The preparation and characterization of nCUR was performed as described in the foregoing sections of this thesis 2.3.2/2.3.5 and 2.3.6.

3.3.3 Experimental design of diabetic cataract model in rats

This blinded study was conducted at National Institute of Nutrition, Hyderabad by our collaborators. The particle preparation and part of the tissue analysis and statistics work were performed at University of Strathclyde. Three months old male WNIN rats with average body weight of 235 ± 15 g (obtained from the National Center for Laboratory Animal Sciences) were used for this study. All the animals were fed AIN-93 diet *ad libitum* throughout the study. The control (Control; n=8) rats received 0.1 M citrate buffer, pH 4.5 as vehicle and the experimental rats received a single intraperitoneal injection of STZ (32 mg/kg) in citrate buffer. After 72 h, blood glucose levels were monitored in rats fasted for 16 h. Animals having blood glucose levels <150 mg/dl were excluded from the experiment and rest were distributed into four groups. Animals in these groups received either only DMSO as vehicle, (Diabetic, D; n=9) or received 2 mg/day dietary curcumin in DMSO, (D+CUR; n=8), 2 mg/day nanocurcumin in water (D+nCUR; n=8) and only blank nanoparticles in water (D+nBLA; n=6) for a period of 10 weeks. Curcumin oral dose (2 mg/day) corresponds to approximately 0.01% curcumin in the diet as used in our previous study (Kumar *et al.,* 2005).

3.3.3.1 Animal care

All experimental procedure was approved by IAEC. The care, use and housing of animals were ensued according to national, institutional guidelines and ARVO statement for use of animals in Ophalamic and vision research. Animals were sheltered individually in separate cages at room temperatiure maintained at 22°C, humidity (50%) and 12h light/dark cycle.

The animals had free access to water. Daily Food intake and body weights (weekly) were recorded. We adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

3.3.3.2 Slit lamp examination and cataract grading

Eyes were examined every week using a slit lamp biomicroscope (Kowa SL15, Portable slit lamp, Tokyo, Japan) on dilated pupils. Initiation and progression of lens opacity was graded into five categories as described previously (Suryanarayana *et al.,* 2003, 2005; Kumar *et al.*, 2005).

Stages of cataract:

Stage 1 – Clear lenses and no vacuoles present

Stage 2 – Vacuoles cover approximately one half of the surface of the anterioi pole forming a subcapsular cataract

Stage 3 – Some vacuoles have disappeared and the cortex exhibits a hazy opacity

Stage 4 – a hazy cortex remained and dense nuclear opacity is present

Stage 5 – a mature cataract present in both cortex and nucleus

3.3.3.3 Collection and processing of blood and lens

Blood was collected through the retro orbital approach from the plexus to estimate glucose and insulin levels after sacrificing the animals by CO_2 asphyxiation and lens anatomized using the posterior approach. They were then stored at 70°C for further analysis. 3-5 lenses were coalesced by homogenizing at 15,000g at 4^0C and 10% homogenate was prepared in pH 7.4 50mM phospate buffer. The soluble fraction of the lens homogenate was

used to analyze the biochemical parameters except the lens sorbitol and malondialdehyde (MDA). These were analyzed in the total homogenate.

3.3.3.4 Biochemical estimations

GOD-POD method was used to analyse the serumglucose using a kit (BioSystems S.A.Costa Brava 30, Barcelona, Spain) and serum insulin by a RIA kit (BRITE-DAE, Mumbai, India) (Suryanarayana *et al.,* 2003, 2007; Kumar *et al.,* 2005). Previously developed methods (Suryanarayana *et al.,* 2005) were applied to estimate lens thiobarbituric acid reacting substances (TBARS), protein carbonyl, AR and sorbitol levels. The specific activity of SOD was assayed according to the reported methods (Suryanarayana *et al.,* 2005). Keeping BSA as standard, the total, soluble and insoluble proteins were determined by Lowry method.

3.3.3.5 SDS-PAGE and size exclusion chromatography of lens proteins

Under reducing conditions and in existence of SDS on 12% polyacrylamide, the cross-linking and subunit profiling of soluble proteins were examined. (Suryanarayana *et al.,* 2005). Size exclusion chromatography on a 600x7.5 mm TSK-G3000 SW column (TOSOH Co., Japan) using a Shimadzu Class-VP HPLC system (Suryanarayana *et al.,* 2005) was used to achieve crystalline distribution in soluble protein fraction. The protein peaks were detected at 280 nm after loading 20 μl of 1mg/ml of soluble protein sample. The flow rate was maintained at 1ml/min using 0.1 M of sodium chloride in 0.1 M sodium phosphate buffer pH 6.7.

3.3.4 Statistical analysis

SPSS version 19.0 software was used for statistical analysis. Descriptive statistics was calculated for all variables. One-way ANOVA was used for comparison of mean values across groups and multiple comparisons were made by Duncan's multiple range test. Whenever heterogeneity of variance was found, non-parametric Mann Whitney "U" test was carried out. Linear regression analysis was carried out to study the relationship for cataract progression with the duration (weeks) and groups (dummy variables). A *p*-value <0.05 was considered significant.

3.4 Results and discussion

3.4.1 Preparation and characterization of nCUR

The nCUR were prepared successfully by previously developed emulsion-diffusion-evaporation technique with slight modification. The Z-average particle size of nanoparticles at 7.5 mg curcumin loading was 282.50±5.72 nm with PDI of 0.14±0.06 resulting in approximately 4.2 mg of curcumin encapsulated (56%) in the particles. The size of blank particles without curcumin was 281.3 ± 8.8 nm with 0.07 ± 0.01 PDI. The freeze dried curcumin loaded and blank particles were comparable to the respective fresh preparations.

3.4.2 Diabetic cataract model in rats

3.4.2.1 Food intake and body weights

The food intake of diabetic animals was more than the respective controls as observed in previous studies (Suryanarayana *et al.*, 2003, 2005; Kumar *et al.*, 2005). Although the food intake was increased (Fig 3.1), the weight of the diabetic animals decreased after concluding the experiment (mean ± S.E body weight at the end of experiment, 205 ± 4.35 g; 162 $p<0.001$) when compared to control animals (320 ± 3.65 g) and the treated groups, CUR, nCUR and nBLA [202 ± 3.15 g, 198±5.22 g, and 164 201± 2.34 g, respectively] did not show any improvement (Fig 3.2).

The characteristics signs of diabetes such as increased intake of food and water and failure to gain weight were observed in STZ treated animals as seen in diabetic subjects.

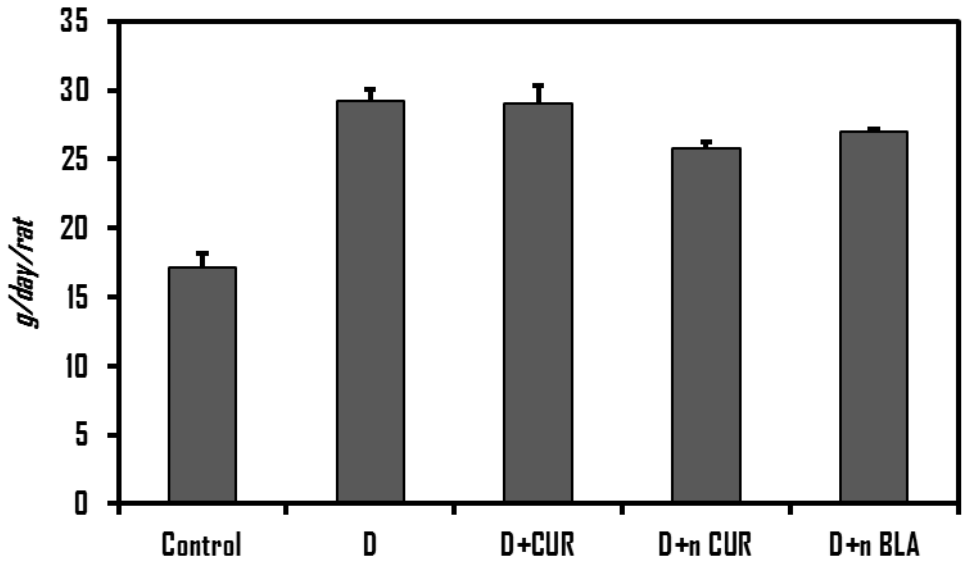

Figure 3.1 Average food intake by rats during the study period. All values represent mean ± sd of the animals in a given group**.**

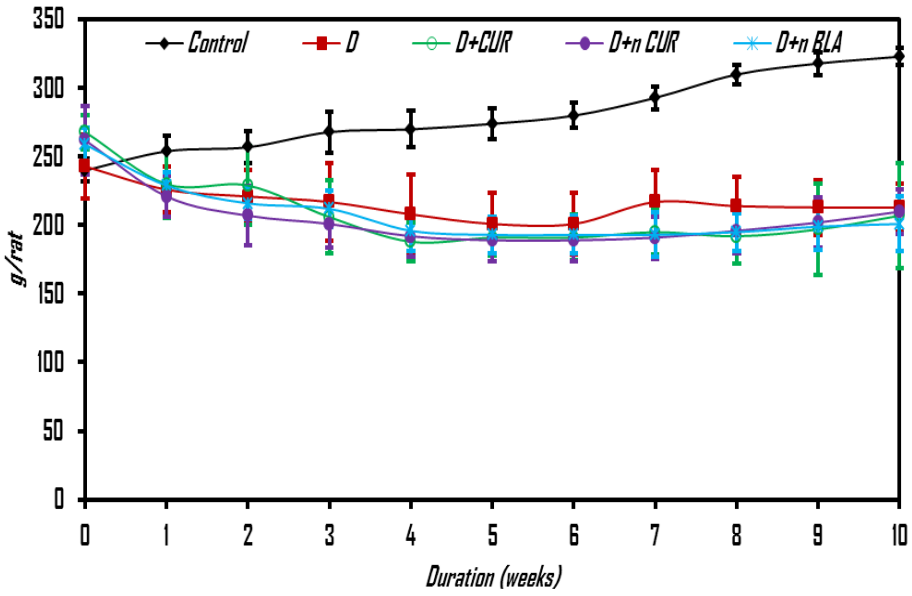

Figure 3.2 Weekly body weight of the animals during the study period**.**

3.4.2.2 Cataract progression

In the untreated diabetic animal group, the cataract progressed and matured by eight weeks after four weeks of STZ injection, feeding of CUR delayed both onset and progression of cataract (β=-0.379; SE=0.080) as compared to untreated group (Figs 3.3 & 3.4) and is consistent with previous observations (Suryanarayana *et al.,* 2005). All the animals receiving STZ developed cataract (100%).The stages of the cataract were averaged at given time and given group as the lenses showed different stages when examined to mainatin an empirical manner in all the groups and presented with duration of diabetes (Figs 3.3 and 3.4). Interestingly, there was a further and significant delay (p<0.001) in the onset and progression of cataract in nCUR group as compared to CUR diabetic group. At the end of ten weeks, the severity of cataract was significantly lower in nCUR group (β=-0.298; SE=0.091) compared to untreated as well as CUR fed diabetic rats. During the experiment, the lenses appeared normal without any changes in opacity in

the control group whereas on the other hand, nBLA did not affect the onset and progression of cataract (β=-0.570; SE=0.080).

The delay in progression of diabetic cataract and enhanced performance of nCUR when compared to CUR can be attributed to improved oral bioavailability and its ability to modulate biochemical mechanisms and development of cataract pathway such as protein insolubilization, oxidative stress, protein glycation, crystallin distribution and polyol pathway. Curcumin at the dose (0.01%) equivalent or slightly higher than the amount of average daily intake (ADI) of turmeric could not prevent diabetic cataract completely, while increase in the dose (>0.01%) maintained similar activity in STZ model, but enhanced the cataract formation in galactose-rat model (Suryanarayana *et al.*, 2003).

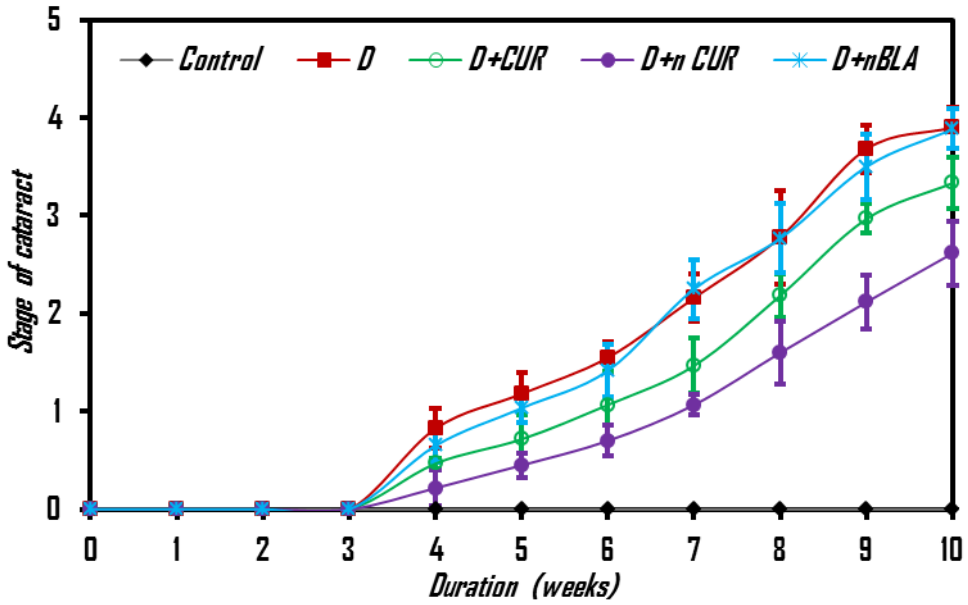

Figure 3.3 The effect of CUR and nCUR on STZ-induced cataract in rats. Stage of cataract in each group was averaged at a given time and the average stage of cataract was plotted as a function of time.

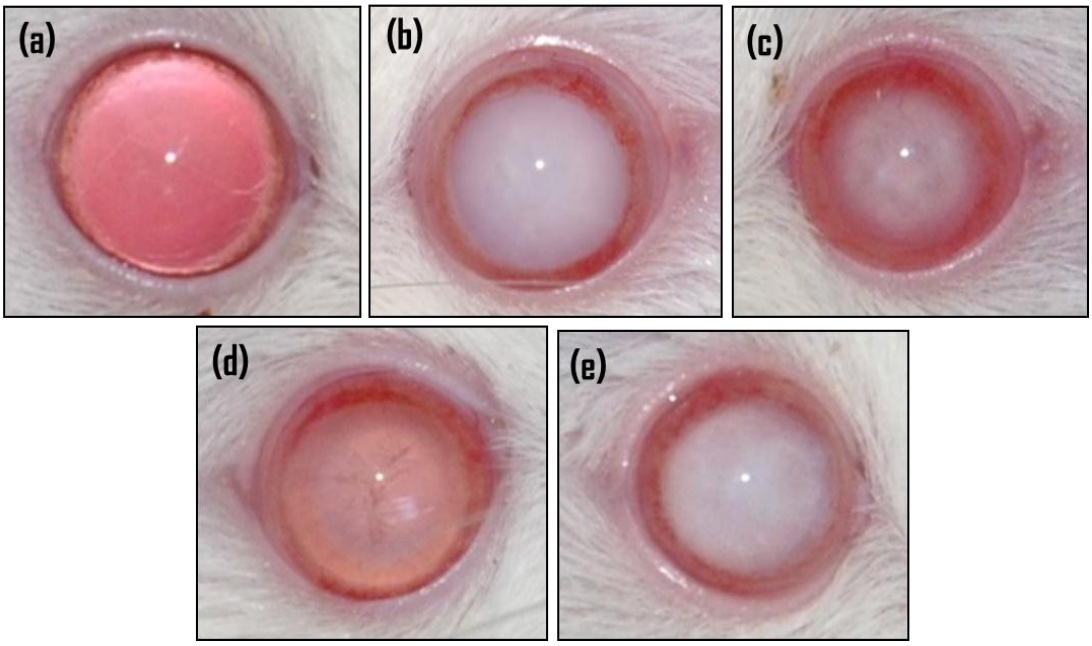

Figure 3.4 Representative photographs of lens from each group (a) control, (b) Diabetic, (c) D+CUR, (d) D+nCUR and (e) D+nBLA at the end of 10 weeks**.**

3.4.2.3 Blood glucose and insulin levels

The blood glucose and insulin levels were estimated to understand if the role of curcumin in cataract onset and progression is due to reduction of STZ-induced hyperglycemia. The blood glucose was elevated as predicted (Fig 3.5) and insulin levels (Fig 3.6) were lowered significantly in untreated group compared to the controls. However, both forms of curcumin also failed to reduce glucose concentrations and increase insulin levels indicating that curcumin's role in delaying cataract progression is independent of its glucose lowering ability.

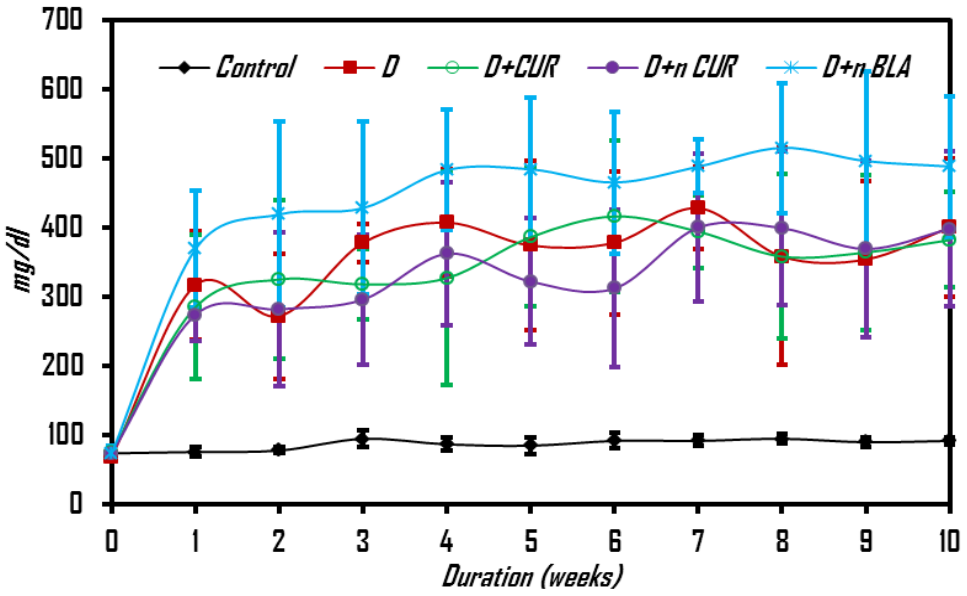

Figure 3.5 The effect of CUR and nCUR on blood glucose in STZ-treated rats compared to non-diabetic control. All values represent mean ± sd of the animals in a given group.

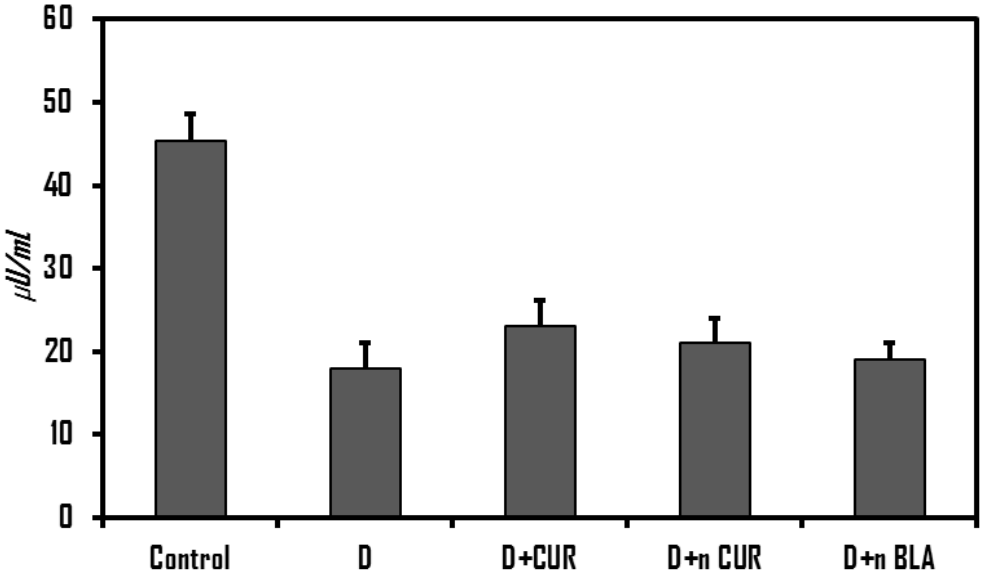

Figure 3.6 Insulin levels of untreated and treated animals compared to non-diabetic controls. All values represent mean ± sd of the animals in a given group.

3.4.2.4 Molecular basis for the delay of cataract

To investigate the effect of nCUR on potential mechanisms in cataractogenesis, biochemical parameters such as protein glycation, oxidative stress, protein oxidation and content, polyol pathway and cystalline distribution were studied.

3.4.2.4.1 Oxidative stress & antioxidant system

Though statistically not significant the rise in MDA levels and protein carbonyl content in diabetic untreated group compared to control group indicates a trend in increased lipid peroxidation and enhanced protein oxidation in the lens (Fig 3.7). Further, altered activity of antioxidant enzyme SOD (Fig 3.7) suggests an elevated oxidative stress in diabetic cataract lens. Both the forms of curcumin prevented the alterations in MDA and protein carbonyls despite raised glucose levels, but not in SOD levels (Fig 3.7). Again the nCUR was more pronounced with respect to MDA levels and protein carbonyl content, though the difference is statistically insignificant.

The protein carbonyls are thought to contribute in general protein malfunction and attributed to oxidative damage although there was no change in SOD activity. Hence, these results suggest oxidative stress may be one of the mechanisms by which curcumin delays the progression and maturation of diabetic cataract and needs further investigations.

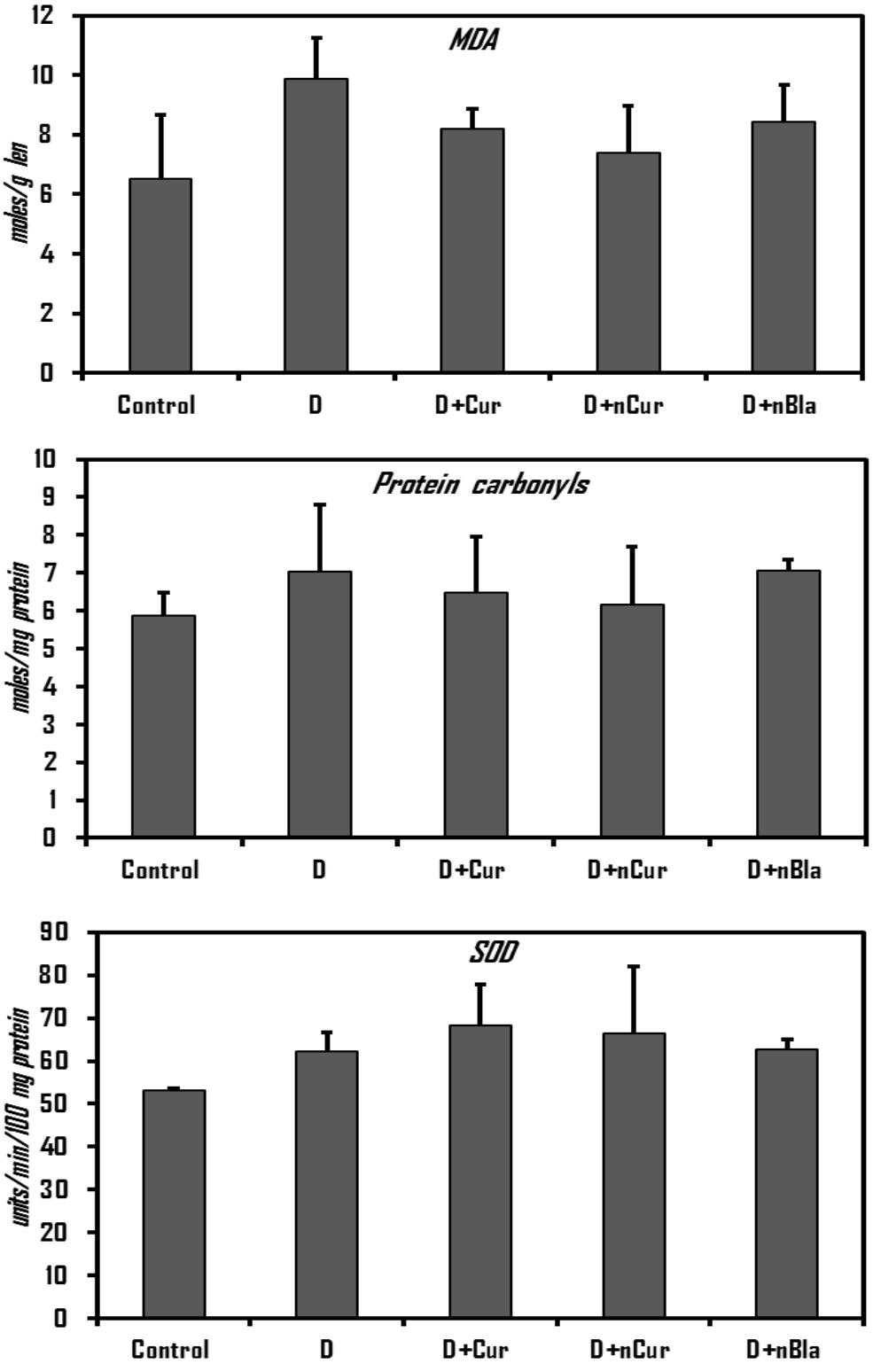

Figure 3.7 The effect of CUR and nCUR on MDA, protein carbonyls and SOD levels in rat lens. The data are mean ± sd (n=4).

3.4.2.4.2 Polyol pathway

While the specific activity of AR, a key enzyme of the polyol pathway was higher, but statistically insignificant in diabetic animals than in control, the increase in sorbitol was remarkably high (Fig 3.8). Treatment with CUR resulted in normalization of AR activity and the effect was prominent with nCUR (Fig 3.8). While, the levels of sorbitol were decreased significantly with CUR, the reduction was highly significant (p<0.001) with nCUR (Fig 3.8). Based on these results, it appears the oxidative milieu plays an important role in AR activity rather than its direct inhibition of AR. Curcumin, particularly nCUR was effective against osmotic stress caused by hyperglycemia.

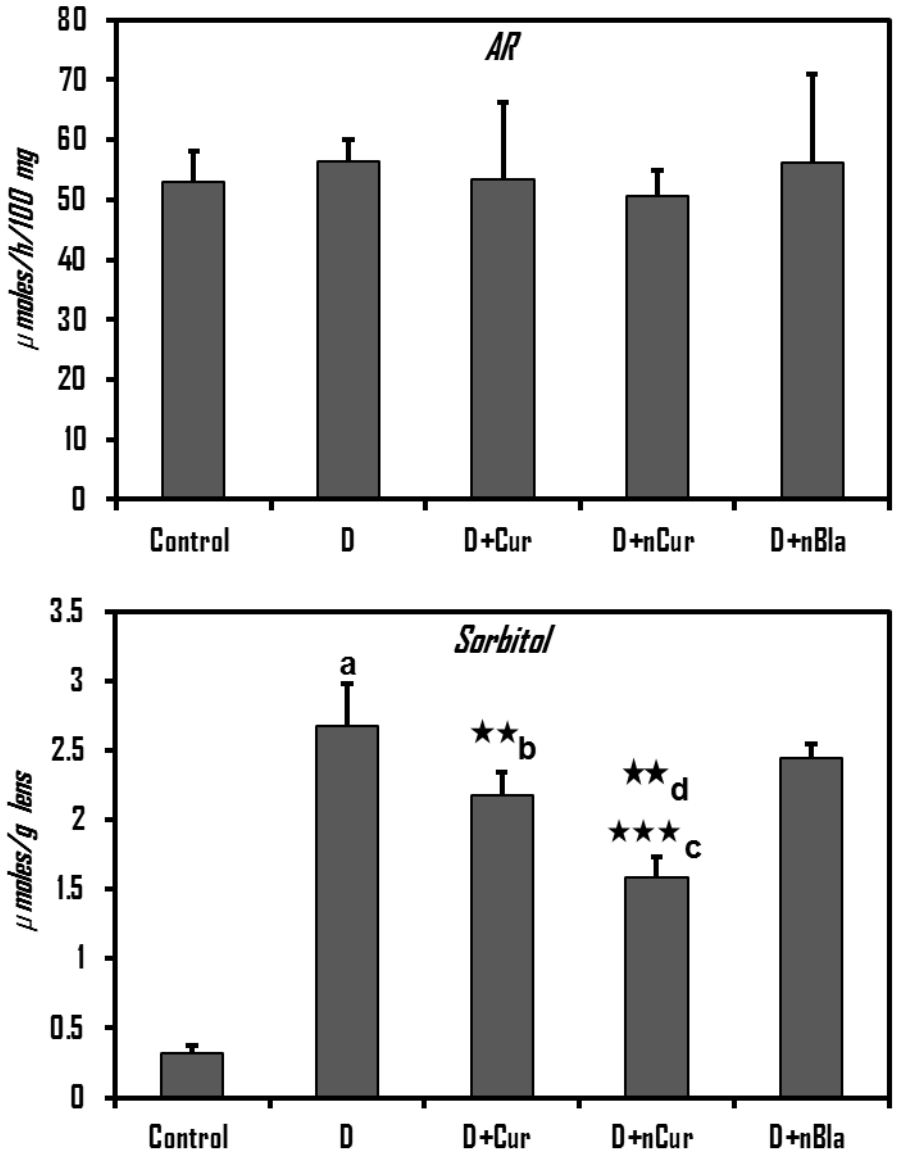

Figure 3.8 The effect of CUR and nCUR on AR activity and sorbitol levels in rat lens. The data are mean ± sd (n=4). $^{**}p<0.01$ a vs b; d vs b & $^{***}p<0.001$ a vs c.

3.4.2.4.3 Protein glycation

To understand whether curcumin feeding to diabetic rats could reduce the glycation, we monitored a few prominent AGE in the lens by immunodetection using specific polyclonal antibodies. The

immunoreactivity of glucose derived AGE, CML and AGE-BSA, in the soluble portion was undetectable in control rat lens, where as these two AGE was significantly detected in untreated diabetic rat lens (Figs 3.9a b). Feeding of rats with CUR and nCUR significantlydecreased the formation of AGE in soluble protein fraction and the effect with nCUR was more prominent than CUR (Figs 3.9 a, b) suggesting the antiglycating activity of curcumin under hyperglycemic conditions.

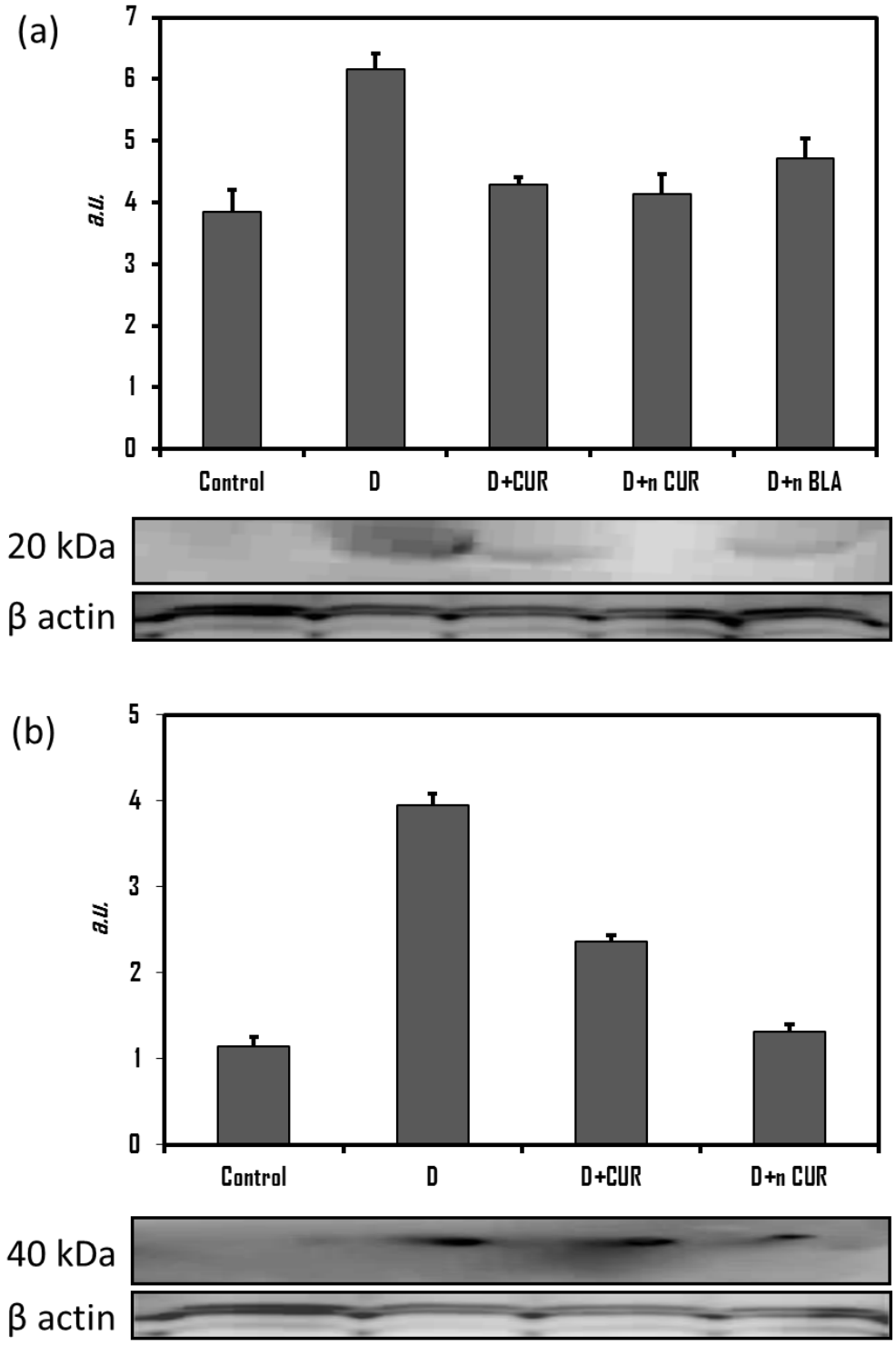

Figure 3.9 Immunodetection of AGE, CML (Panel a) and AGE-BSA (Panel b), in the soluble protein fraction of rat lens. The data in bar diagram are mean ± sd (n=3) and the blot is a representative of three independent analyses.

3.4.2.4.4 Crystallin distribution, protein aggregation and insolubilization

Whatsoever, the conclusive result of lens opacification is considered due to insolubility and alteration in protein profiling of otherwise soluble proteins. Therefore, we have analysed the total, soluble and insoluble protein content in all the groups. The levels of soluble and total proteins was significantly lower (p<0.05) in the diabetic group in comparsion to control group (Fig 3.10). During progression and subsequent maturation of cataract, there is an insolubilization of the available soluble proteins in addition to the leakage of proteins from lens because of osmotic stress and degradation of the proteins in lens causing reduction in the levels of protein cotent. Dosing the rats with CUR improved the total and soluble protein levels (p<0.05), correlating with the delay of onset of cataract in this group (Fig 3.10). However, it should be noted that the improvement in percentage of soluble protein with nCUR was remarkable compared (p<0.001) to CUR (Fig 3.10).

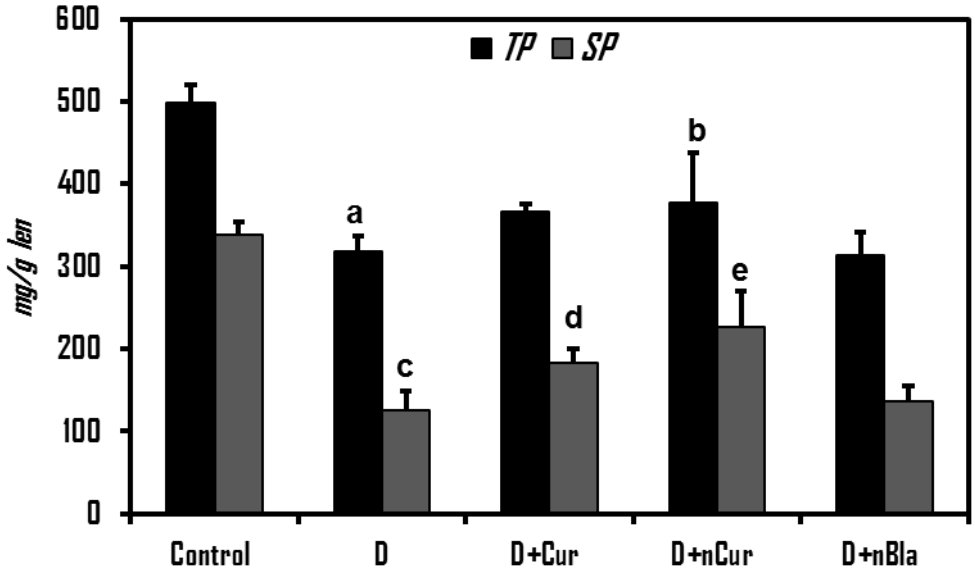

Figure 3.10 The effect of CUR and nCUR on protein content of rat lens. The data are mean ± sd (n=6). *p<0.05 a vs b, c vs d; ***p<0.001 c vs e.

The soluble proteins were investigated using size exclusion chromatography to further inspect the variation in crystallin profile and this showed a decline in β- and γ-crystallin abundance while an increase in α-crystallin peak along with to appearance of a high molecular weight (HMW) aggregates peak in the void volume in diabetic untreated compared to control (Fig 3.11). The decrease in β- and γ-crystallins suggest protein modifications/disintegration in diabetic cataract lens, which may be involved in the formation of HMW aggregates (including increase in α-crystallin peak) because of aggregation, crosslinking or other alterations (Fig 3.11). CUR group showed improved crystallin distribution pattern and nCUR showed crystallin profile comparable to that of control rats (Fig 3.11). Further, the cross-linking of lens protein and HMW aggregates were observed by SDS-PAGE pattern of soluble protein. The diabetic rat lens when compared to the control and CUR groups, demonstrated elevated ratio of cross-linked and aggregated proteins

with presence of non-disulphide dimers of molecular weight 45kDa. (Fig 3.12).

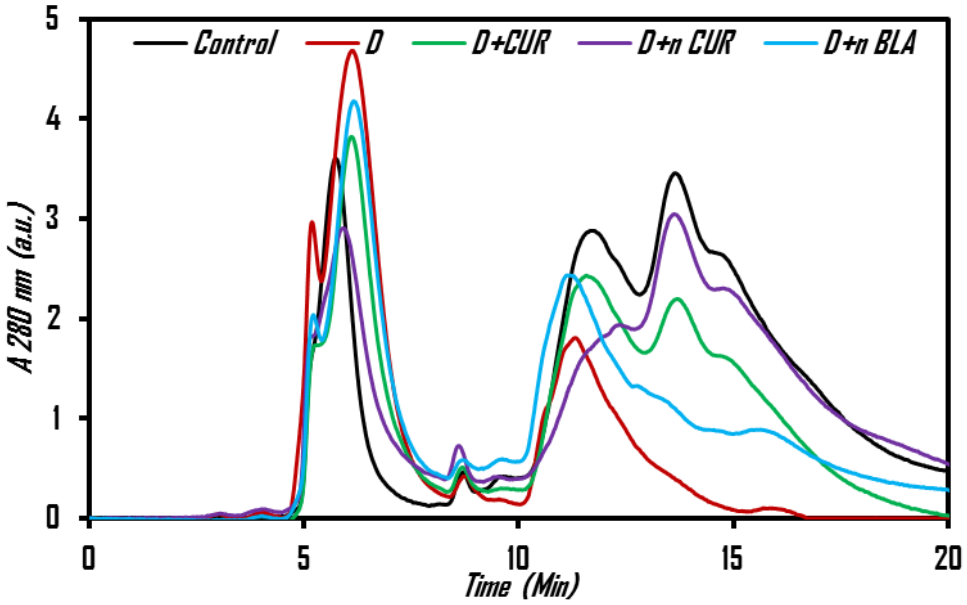

Figure 3.11 The effect of CUR and nCUR on crystallin distribution in the soluble protein fraction of rat lens. Soluble protein was loaded on TSK-G3000 SWXL gel filtration HPLC column and protein peaks were detected at 280 nm. Peaks representing α-, β- and γ- crystallins are indicated at their respective positions.

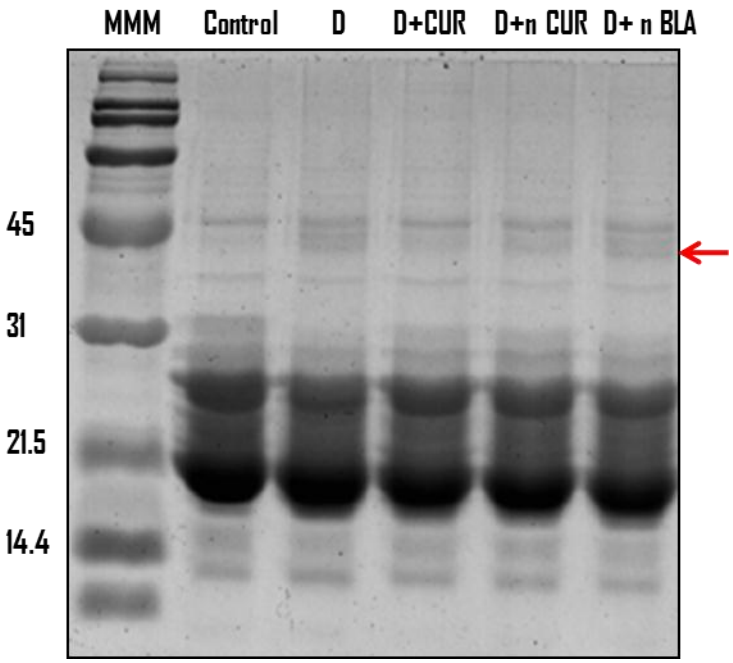

Figure 3.12 The effect of CUR and nCUR on protein crosslinking and the aggregation of the soluble fraction of the lens. Soluble protein was loaded onto a polyacrylamide gel. Molecular mass markers (MMM); Arrow: cross-linked proteins.

3.5 Conclusion

- nCUR was superior to CUR in delaying the progression of the cataract.
- The superior activity of nCUR can be attributed to improved oral bioavailability resulting in better management of oxidative stress/antioxidant system, polyol pathway, protein glycation, protein oxidation, protein content, and crystallin distribution that are involved in the development and progression of cataract in diabetes.

4 NEUROVASCULAR PROTECTION BY nCUR IN A MURINE MODEL OF EMBOLIC STROKE

4.1 Introduction

Stroke is a cerebrovascular (CVA) incidence. It is characterised by the loss of blood supply to the brain, leading to vascular dysfunction and neuro-glial cell death, and subsequently, loss in brain functions. Majority of the stroke is embolic in nature (ischaemic stroke) and could be hemorrhagic too (rupturing of blood vessel causing bleeding and subsequent ischeamia) (NICE Clinical guidance 68, 2008). Ischaemic stroke accounts for ~80% of strokes. Approximately 40% of them remain undefined for the cause.

Approximately 15-million stroke incidence occurs every year worldwide. In UK, stroke is a major cause of the adult morbidity and disability. It is the second most common cause of mortality globally. Even though the prevalence of stroke has increased since 1999 (Lee *et al.,* 2011) the incidence and mortality rate has declined due to improved medical emergency care (Rosamond *et al.,* 2008; Lee *et al.,* 2011) in UK and US. In 2007, The British Heart Foundation and The Stroke Association jointly estimated that stroke is third most common cause of death in England after heart disease and cancer accounting for about 53,000 deaths every year (Scarborough *et al,* 2009) and 150,000 people diagnosed every year. According to WHO classification of World Bank's Country classification of economies, two country groups i.e. high income countries and low to medium income countries from 1970 to 2008 (for four decades) and types of stroke depending on pathology of stroke (when possible) were considered. From this study there was a reduction in stroke incidence rate in high income countries and increase in the low to medium income countries (Feigin *et al.,* 2009).

Several non-modifiable risk factors have been identified and associated with increased risk of stroke where age is the most important factor, while hypertension is considered as a modifiable risk factor (Martin *et al.,* 2010; Endres *et al.,* 2011). It is also believed that lifestyle such as diet, smoking and exercise also plays some role.

To date, the only FDA approved treatment for AIS is intravenous tissue plasminogen activator (tPA) that activates the endogenous fibrinolytic system and facilitates thrombolysis (Schellinger *et al.,* 2001; Lapchak, 2002). The current first line treatment tPA requires administration within 3 h after the onset of the symptoms for two obvious reasons that it will be ineffective or can increase the risk of bleeding in the brain when used 3 h post-stroke (Hacke et al., 2008). Limited therapeutic window of tPA therapy leaves over 95 percent of ischemic stroke patients without an effective intervention. Because of this dismal reality, identification of treatment strategies that can be used safely post-ischemia, and can possibly reduce the reperfusion injury in combination with IV-tPA therapy or surgical interventions, is critical.

AIS are caused due to cascade of events following occlusion of blood vessel (Dirnagl *et al.,* 1999; Lapchak & Araujo, 2007). Therefore, post-stroke pathophysiology is complex due to its neurovascular origin. Oxidative stress is considered as a major component in the progression of post-stroke pathophysiology (Facchinetti *et al.,* 1998; Love, 1999). A free radical scavenger, Edaravone, is a low molecular hydrophobic and lipophilic compound that has ability to cross BBB. Edaravone exhibits its activity through its antioxidant activity by scavenging the hydroxyl, peroxyl and superoxide radicals but also activates other pathways (Lapchak, 2010). It is currently being studied for its safety and pharmacokinetic profiling in patients (ClinicalTrials.gov, 2009). The NeuroThera Effective and Safety Trial

(NEST)-1 and (NEST)-2 Trials investigated the use of Transcranial near infrared laser therapy (NILT) and shown as a possible neuroprotective treatment for AIS (Lapchak, 2010). Currently, a number of endovascular approaches have been investigated and reported advantages over tPA and have continued to evolve (Nogueira *et al.,* 2009). Curcuminoid CNB-001 and minocycline that acts by pleiotropic activity are being implicated as treatment options for stroke (Fagan *et al.,* 2011; Lapchak, 2011) in both *in vitro* and *in vivo*.

Many strategies for neuroprotection have been found promising in pre-clinical studies, but failed in clinical trials. Hence to improve the service and make treatment accessible, telestroke has a promising role maximizing the effective management to larger group of patients through telecommunication (Boy, 2012). While research is on for a safer alternate therapy after stroke for neuroprotection or neurovascular protection, safe extension of the effective therapeutic window of tPA therapy is also a high priority research.

4.1.1 Stroke models

A number of experimental models of stroke have been developed for both translational and mechanistic studies to understand the post-stroke pathophysiology to develop novel treatment strategies. Most of the research is being carried in rats due to several advantages apart from low cost and ethical issues (Takizawa *et al.,* 1991; Macrae, 1992; Brinker *et al*., 1999) based on suture occlusion models where the reperfusion-based injury dominates. Basically two different pathophysiologies after stroke exist, which depend primarily upon the severity of the cerebral blood flow (CBF) reduction and later on the dynamics of CBF restoration (Thiyagarajan & Sharma, 2004;

Szabo *et al*., 1995) the former being prevalent in most of the human cases. Therefore, post-stroke pathophysiology in humans is mostly ischemia dependent but not often reperfusion dependent. The sudden reperfusion as in mechanical suture middle cerebral artery occlusion-reperfusion (sMCAO) model seldom happens in human stroke. Most of the human cases either remain non-reperfused or partially reperfused due to self-recanalization. Therefore, thromboembolic stroke model, achieved by delivering a clot in the brain, offers greater advantages over the other models (Brinker *et al.,* 1999; Zhang *et al.,* 2004). It is evident that no single animal model can mimic the circumstances completely and therefore the Stroke Therapy Academic and Industry Roundtable (STAIR) put forward a recommendation that the therapies should be tested in different models, sexes and under various comorbid conditions (Fischer *et al.,* 2009).

4.2 Specific aims

- To evaluate and assess the potential and pharmacodynamics of curcumin loaded PLGA nanoparticles in embolic stroke model in mice. Since most of the human stroke is embolic in nature and remain non-reperfused/ untreated with tPA, in this preliminary study, we tested nCUR after embolic stroke in mice which are untreated with tPA.

4.3 Materials and Methods

4.3.1 Materials

HRP conjugated donkey anti-rabbit IgG antibody was obtained from Jackson ImmunoResearch, West Grove, PA, Anti-MMP-9 and MMP-2 antibodies

were obtained from Cell Signaling Technology Danvers, MA. Anti-β–actin antibody and TTC were purchased from Sigma-Aldrich Co. ABgene reagents were procured from Fisher scientific. EZQ® Protein Quantitation Kit, NuPAGE® Novex® Bis-Tris gels and DHR123 were purchased from Invitrogen. FITC-labeled gelatin, Lysis-M EDTA-free buffer and PVDF membranes were purchased from Molecular Probes, Eugene, OR ; Roche Diagnostics, Indianapolis, IN and Millipore Billerica, MA respectively. Probe 407 was purchased from Perimed Inc., Sweden.

4.3.2 Preparation and characterization of nCUR

The preparation and characterization of nCUR was performed as described in the foregoing sections of this thesis 2.3.2/2.3.5 and 2.3.6.

4.3.3 Experimental design of stroke model in mouse

4.3.3.1 Animals and Experimental Groups

This blinded experimental procedure was conducted and approved by The Institutional Animal Care and Use Committee (IACUC) of Georgia Regents University (GRU) as per the National Institute of Health (NIH) guidelines. C57BL/6J wild type male mice (20 ±1 weeks old; Jackson Laboratory, Bar Harbor, Maine; Stock# 000664) housed in the GRU's Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) accredited facility were used in the experiments. The groups used in the study were as follows: Sham, (sham surgery-operated vehicle treated mice); Stroke+Veh (eMCAO stroke injury followed by vehicle

treatment); and Stroke+nCUR (eMCAO stroke injury followed by nCUR treatment).

4.3.3.2 Preparation of clot/ emboli

Clot was prepared as modified by us earlier (Hoda *et al.,* 2011; 2012). The mouse arterial blood was supplemented along with human fibrinogen 2mg/ml) and instantly clotted with PE-50 tube for 6h at 25°C and then stored at 4°C for a day to improve the stableness and uniformity of the fibrin core of the clot. Before the use, clot (~5 cm) was extruded and briefly washed with sterile deionized water into a Petri dish. The fibrin rich core was obtained and transferred into another Petri dish containing sterile phosphate-buffered saline (PBS) and left for further retraction at room temperature for 3-4 h.

4.3.3.3 Embolic stroke model

It was performed as reported earlier by our collaborators (Hoda *et al., 2011; 2012*). Mice were sedated with buprenorphine (0.05 mg/kg body weight) as analgesic, and anesthetized with 3.5% isofluorane and maintained with 1.5–2.0% during the surgery. Body temperature was maintained at 37 °C by a thermo-regulated surgery pad. A 1-cm long incision was made into the skin dorsal to the head. The pre-ischemic level of the cerebral blood flow (CBF) was recorded using a laser Doppler flow meter (PeriFlux 5001 system). The internal carotid artey, right common carotid artey and the external carotid artey were assessed by a midline incision on ventral side of the neck. A temporary atraumatic clip was placed on the CCA to prevent loss of blood during catheter insertion. A modified PE-10 catheter containing a single

fibrin rich clot (9 ±0.5 mm length) was introduced into the ECA and advanced into the ICA. The clot was gently injected with 100 μl of PBS. Catheter was removed immediately after embolization and the arterial wound was secured to prevent blood loss. Temporary clip on the CCA was removed and the blood flow was restored therein. The drop (>65% as compared to the pre-ischemic value) in the CBF in the MCA region was confirmed using laser Doppler flow meter. The site of the surgery was closed.

4.3.3.4 Treatment Protocols

All the treatments (Vehicle control or nCUR) were injected intraperitoneal (IP) as suspension in sterile PBS immediately (0 h) after the surgery (sham or stroke procedures) and 3 h post-surgery. Mice were treated according to the groups as follows: Sham group with the control vehicle containing curcumin free nanoparticles (nBLA) equivalent to nCUR; Stroke+Veh group with nBLA; and Stroke+nCUR with 10 mg/kg body weight of nCUR.

4.3.3.5 Magnetic Resonance Imaging (MRI)

Non-invasive MRI was performed using a horizontal 7.0T BioSpec MRI spectrometer (Bruker Instruments) equipped with an 8.9 cm micro-imaging gradient insert (100 gauss/cm). Anesthetized mice were positioned with the MR scanner. Breathing was controlled at 35 respirations/min and core body temperature was maintained at 37 °C using a recirculating water bath. High-resolution T2-weighted (T2W) images were acquired, using a surface coil developed in-house.

4.3.3.6 Bederson Neurological Assessment and Score

Neurological deficits were assessed on a modified 5-point Bederson scoring scale at 24 h post-stroke as adopted by us earlier (Hoda *et al*., 2011): 0, no deficit (normal behavior as in sham); 1, deficit of foreflexion on contralateral side; 2, weaken resistance to lateral push with torso turning to the ipsilateral side when gripped by the tail; 3, very significant circling towards the affected side; and 4, barely moves and prefer to rest or lay down on the bed.

4.3.3.7 Adhesive Tape Removal Test

This test helps to detect sensorimotor deficit, and was performed as reported earlier with slight modification (Bovet *et al*., 2009; Zhao *et al*., 2006). Briefly, naïve mice were acclimatized for 3 days prior to surgery by placing them in a transparent Perspex box (15 cm x 25 cm). Two pieces of adhesive tape (0.3 cm x 0.4 cm) were used as bilateral tactile stimuli attached at the distal-radial region on each forelimb such that they cover the hairless part (3 pads, thenar and hypothenar). Within 180 seconds, the tape removal time was recorded as the function of sensorimotor outcome. Mice were scored 180 seconds for removal time if they failed to remove the tape.

4.3.3.8 Infarct analysis

Infarct analysis was performed as reported by our collaborator (Hoda *et al.*, 2011; 2012). Briefly, brain was harvested after perfusion sacrifice with chilled phosphate-buffered saline (PBS; 0.01 M) under deep anesthesia. 1-mm thick coronal sections were prepared. The alternate coronal sections were stained

with 2% 2, 3, 5-triphenyltetrazolium chloride (TTC) and were fixed with 10% formalin in PBS. The images were digitalized, and the infarct volume was analyzed using Scion Image analysis software (Scion Corporation).

4.3.3.9 Histopathological

Hematoxylin and eosin (H & E) staining was used for the histopathological evaluation of the brain after stroke. Briefly, brain tissue was fixed in 4% paraformaldehyde 24 h prior to processing and embedding in paraffin wax. 7 um sections were prepared and de-paraffinized with xylene and ethyl alcohol (100 %, 90 % and 70%). Sections were stained with hematoxylin (blue color) and eosin (pink color) for nuclei and cytoplasm, respectively.

4.3.3.10 Isolation of RNA, synthesis of cDNA, and real-time PCR

Real-time PCR was performed as cited and described earlier with some modifications (Hoda *et al*, 2009). Snap frozen brain tissues were homogenized and suspended in Trizol reagent for RNA isolation. The isolated RNA under manufacturers guidelines (Gibco BRL, Carlsbad, CA) was observed for the quality at absorbance 260 and 280 nm (Helios-Gamma Thermo Spectronic, Rochester, NY). RNA was isolated following manufacturer's (Gibco BRL, Carlsbad,CA) instructions and the quality of the RNA preparation was monitored by absorbance at 260 and 280 nm (Helios-Gamma, Thermo Spectronic, Rochester, NY). RNA was reverse-transcribed into complementary deoxyribonucleic acid (cDNA) using iScript reagents from Bio-Rad on a programmable thermal cycler (PCR-Sprint, Thermo Electron, Milford, MA). 50 ng of cDNA was amplified in each real-time PCR using a

Bio-Rad iCycler, ABgene reagents and appropriate primers (Table 1). For normalization Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control

Table 4.1 Nucleotide sequences of mouse primers used for RT-PCR

Gene	Primer	Reference/Accession Number
IL-1β	GCA CCT TAC ACC TAC CAG AGT	NM_010554.4
	AAA CTT CTG CCT GAC GAG CTT	
IL-6	TAG TCC TTC CTA CCC CAA TTT CC	NM_031168.1
	TTG GTC CTT AGC CAC TCC TTC	
iNOS	GAT GTG CTG CCT CTG GTC TT	NM_010927.3
	TT GGG ATG CTC CAT GGT CAC	
TNF-*α*	CCC TCA CAC TCA GAT CAT CTT CT	NM_013693.2
	GTC ACG ACG TGG GCT ACA G	
GAPDH	CAT GGC CTC CAA GGA GTA AGA	M32599
	GAG GGA GAT GCT CAG TGT TGG	

4.3.3.11 Measurement of plasma peroxynitrite

In a separate set of experiments with a 6 h end-point, we measured plasma peroxynitrite by using a fluorescent probe dihydrorhodamine 123 (DHR123; Invitrogen) as reported earlier with certain modifications (Szabo et al., 1995; Thiyagarajan & Sharma, 2004; Hoda *et al., 2012*). DHR123 is oxidized to rhodamine 123 (Rhod123) in a peroxynitrite dependent manner. DHR123 was injected bolus (4μmol/kg in 0.2 ml saline) after 3 h post-stroke. Plasma was collected at 6 h post-stroke (3 h after DHR123 injection), the fluorescence was measured (λ_{ex} 500 nm, λ_{em} 536 nm).

4.3.3.12 Evaluation of blood brain barrier (BBB) disruption by Evan's blue (EB) extravasation

BBB leakage was assessed by the method of Weismann and Stewart and as reported previously by our collaborators with slight modifications (Hoda *et. al.,* 2009). Six hours before sacrifice, mice were injected intravenously with 100 µl of a 5% solution of EB in saline. Under anaesthesia, cardiac perfusion was conducted using 100 ml of saline thus clearing the circulation of EB from the cerebral hemispheres.The brain was dissected, segmented and photographed. After isolation of the two hemispheres, they were homogenised in 250 µl of N, N-dimethylformamide (DMF). The suspension obtained was kept at room temperature in the dark for 72 h. It was centrifuged at 10, 000 x g for 25 min, and the supernatant was spectrofluorimetrically analyzed (λ_{ex} 620 nm, λ_{em} 680 nm).

4.3.3.13 Measurement of edema

It was performed in a set of separate experiments with an end-point of 24 h post-stroke. To determine the brain water content, the animals were euthanized as reported earlier (Hoda *et al.,* 2009). The cortices, the ipsilateral and the contralateral hemispheres excluding the cerebellum were dissected carefully and weighed individually. The dry weight of each of the hemispheres was determined after they were dried at 60 °C for 72 h. The water content was calculated in ipsilateral hemisphere using the formula

$$water\ cotent\ (\%) = \frac{wet\ weight - dry\ weight}{wet\ weight} \times 100$$

4.3.3.14 In situ zymography

In situ zymography was performed to detect and localize MMPs (gelatinolytic) enzyme activity in tissue sections as reported earlier (Lee *et al.*, 2004). This does not differentiate between MMP-2 and MMP-9 but detects regionally specific gelatinolytic activity. Gelatin with a fluorescent tag remains caged (no fluorescence) until the gelatin is cleaved by gelatinase (MMPs) activity. In brief, mice were transcardially perfused with ice-cold PBS (pH 7.4) 6 h post stroke, their brains were immediately harvested and frozen in 2-methylbutane with liquid nitrogen without fixing them. The sections (20 μm) obtained were incubated overnight in 0.05 M Tris-HCl, 0.15 M NaCl, 5 mM $CaCl_2$, and 0.2 mM NaN_3, pH 7.6, containing 40 μg of FITC-labeled gelatin at room temperature. The *in situ* gelatinolysis was revealed by the appearance of fluorescent brain constituents and were imaged.

4.3.3.15 Immunoblotting

Brains were harvested as explained above 6 h post-stroke. To analyze the total proteins, the ischemic hemispheres were homogenised and disintegrated in complete lysis-M EDTA-free buffer using the EZQ® Protein Quantitation Kit. As reported earlier (Hoda *et al*, 2011), the samples (30 μg) were subjugated to SDS-PAGE using 10% NuPAGE® Novex® Bis-Tris gels prior to their transference through 0.2 μm PVDF membranes. Membrane was blocked for non-specific binding (5% BSA solution), and incubated with polyclonal anti-MMP-9 and MMP-2 antibodies at 4 °C overnight, followed by HRP conjugated donkey anti-rabbit IgG antibody. Membranes were re-probed with mouse monoclonal anti-β–actin antibody as a loading control.

Visualisation of the proteins was conducted with the ECL detection system (Pierce, Thermo Fisher Scientific) on autoradiography film (Denville Scientific, Metuchen, NJ). Image-J software was used to scan and process images to measure the densitometry by an investigator blinded to the groups.

4.3.3.16 Post-stroke survival study

Mice (n = 10 per group) were assigned to different groups and were allowed to survive for a week post-surgery (sham or stroke). They were injected intraperitoneal either with vehicle control or nCUR suspension at 0 and 3 h post-surgery, followed by every 24 hours for 3 days. During the survival, mice were supplemented with 1 ml of lactated Ringer's solution subcutaneously twice daily and NAPA gel as electrolyte and food supplements, respectively. The mortality and survival were recorded daily.

4.3.4 Statistics

SAS® 9.2 software was used for statistical analysis. All data are expressed as mean ± SD. One-way ANOVA was used for comparison of mean values and multiple comparisons were made using Tukey's multiple comparison tests. T-test was applied wherever required and appropriate. Statistical significance was determined at $P < 0.05$.

4.4 Results and discussion

4.4.1 nCUR decreases injury and improves behavioral outcomes after stroke

As shown in the representative coronal and axial MR images in Fig 4.1 and Fig 4.2, eMCAO produces significant injury in the vehicle treated mice ~16 hours post-stroke while the nCUR treatment apparently reduced the core formation (white area) after stroke. Therefore, we anticipated that nCUR should provide neurobehavioral benefits, and evaluated them ~20 h post-stroke. MRI being more sensitive technique was carried out on representative animals to assure and confirm the localization of the stroke.

First, we blindly scored the mice of the two groups for the neurological deficit score (NDS) as a function of their motor deficit and appearance; normal being 0 (zero). As evident from Fig 4.3 mice from Stroke+Veh group showed poor score (mean NDS = 3.0) while Stroke+nCUR was comparatively better (mean NDS = 1.5). Post-stroke nCUR treatment significantly attenuated the neurological deficit by 50% ($p \leq 0.01$) as compared to the vehicle treatment. Moreover, we evaluated the sensorimotor function of mice from different groups as an additional neurobehavioral outcome after stroke (Fig 4.4). As compared to the basal values (sham), there was a very significant deterioration in the sensorimotor function in Stroke+Veh group ($p \leq 0.001$). Treatment with nCUR significantly improved the sensorimotor function as compared to the vehicle treatment to the stroke group ($p \leq 0.001$).

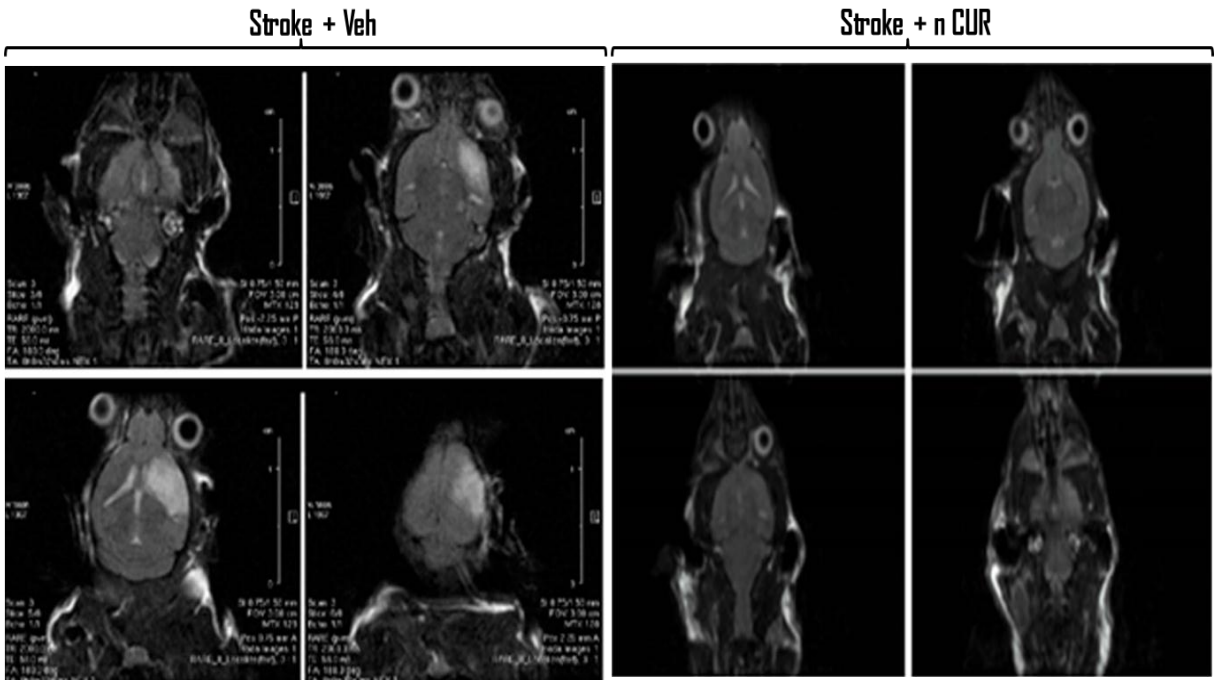

Figure 4.1 Coronal view of the brain imaged using MRI**.**

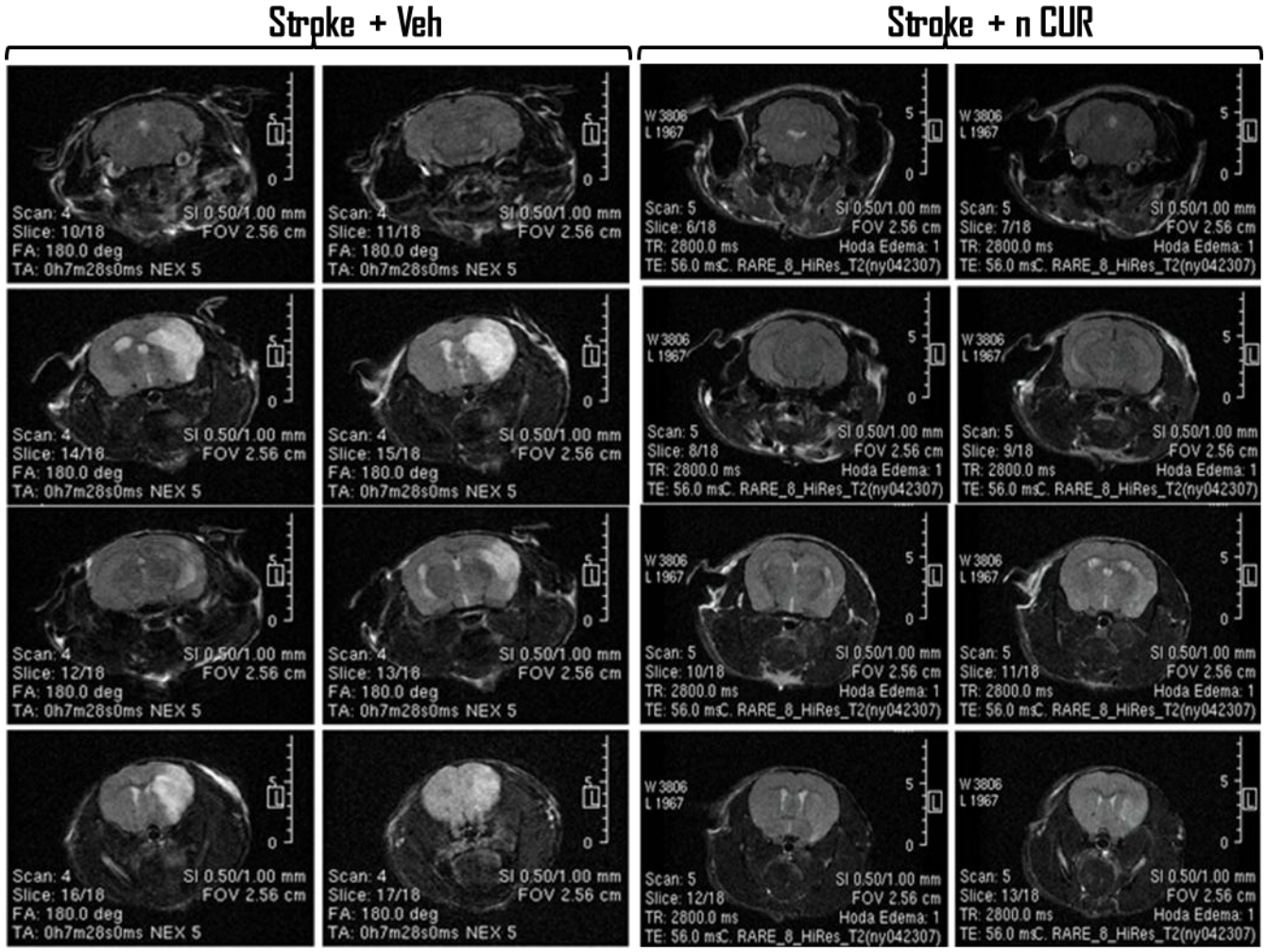

Figure 4.2 Axial view of the brain imaged.

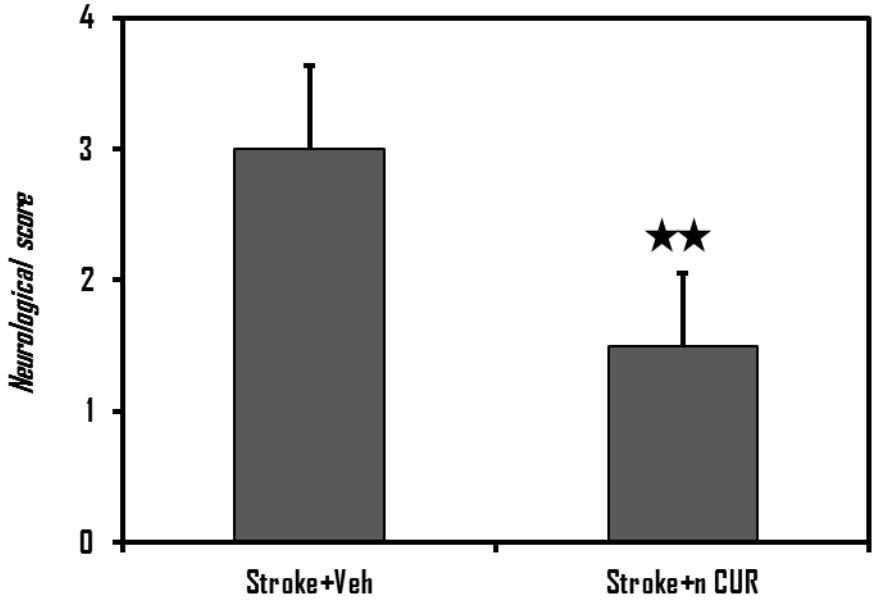

Figure 4.3 Neurological status of the mice assessed by a person blinded to the treatment. All values represent mean ± sd of the animals in a given group (n=6).

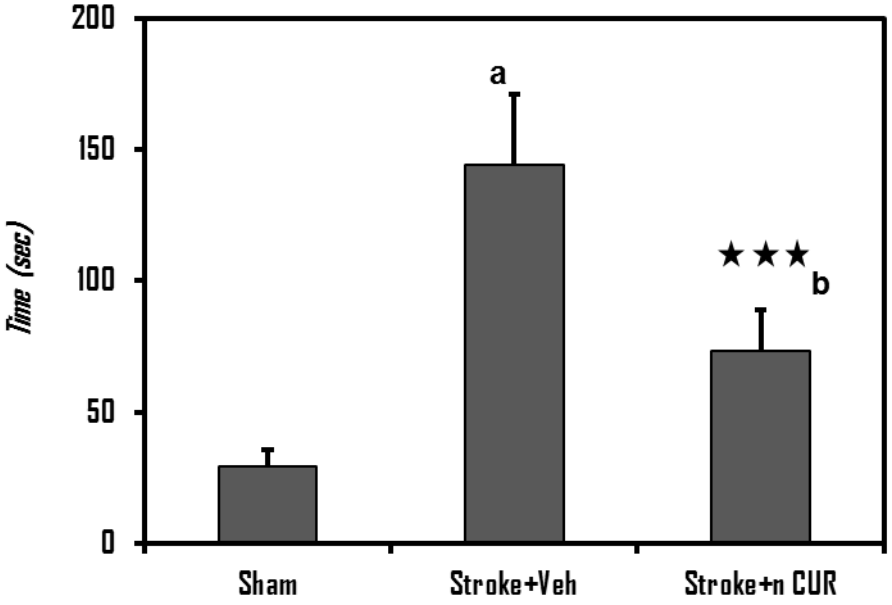

Figure 4.4 Sensorimotor function of the mice. All values represent mean ± sd of the animals (n=6) in a given group. ***p<0.001 a vs b (n=6).

4.4.2 nCUR decreases post stroke infarction and cell death

Figs 4.5 – 4.6 show the post-stroke infarction and its attenuation by nCUR treatment. Sham group never showed any injury after TTC staining. As evident from the representative TTC images, Stroke+Veh group had a larger core area (~52%) as compared to the nCUR treated mice (~38%). When compared, nCUR treatment significantly decreased the infarction volume (~26%; $p \leq 0.01$) than vehicle treatment at 24 h post-stroke. Representative H & E images (Fig 4.7) further supported that nCUR blocked the post-stroke neuronal cell death; thus attenuated the infarction.

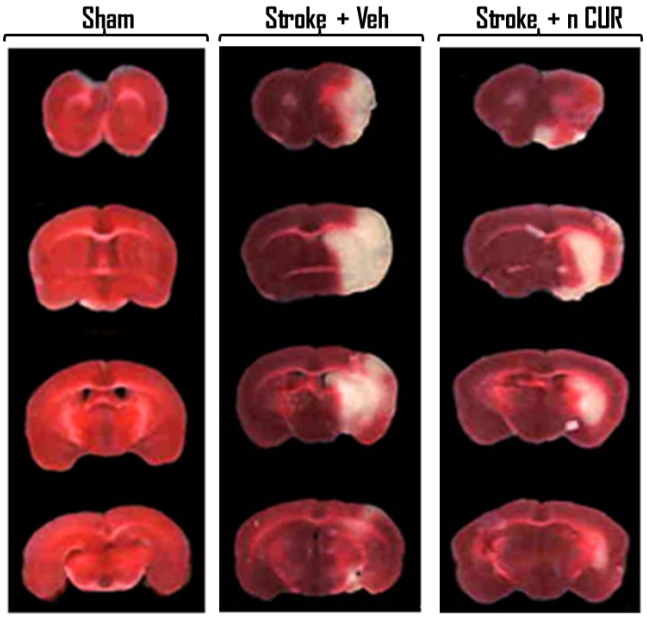

Figure 4.5 Coronal sections stained with TTC to analyze the infarct volume**.**

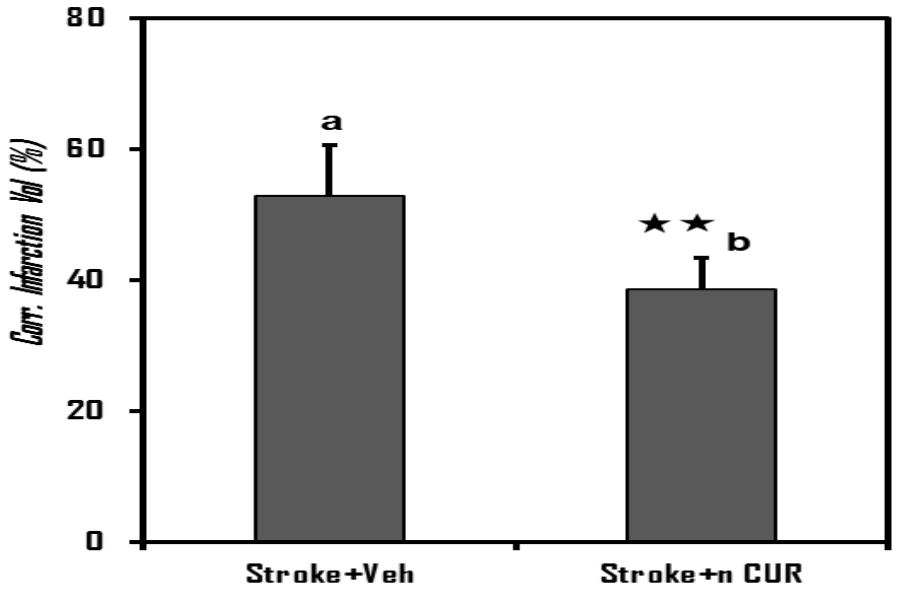

Figure 4.6 Infarct volume significantly reduced in nCUR treated. All values represent mean ± sd of the animals in a given group (n=6). **p<0.01 a vs b.

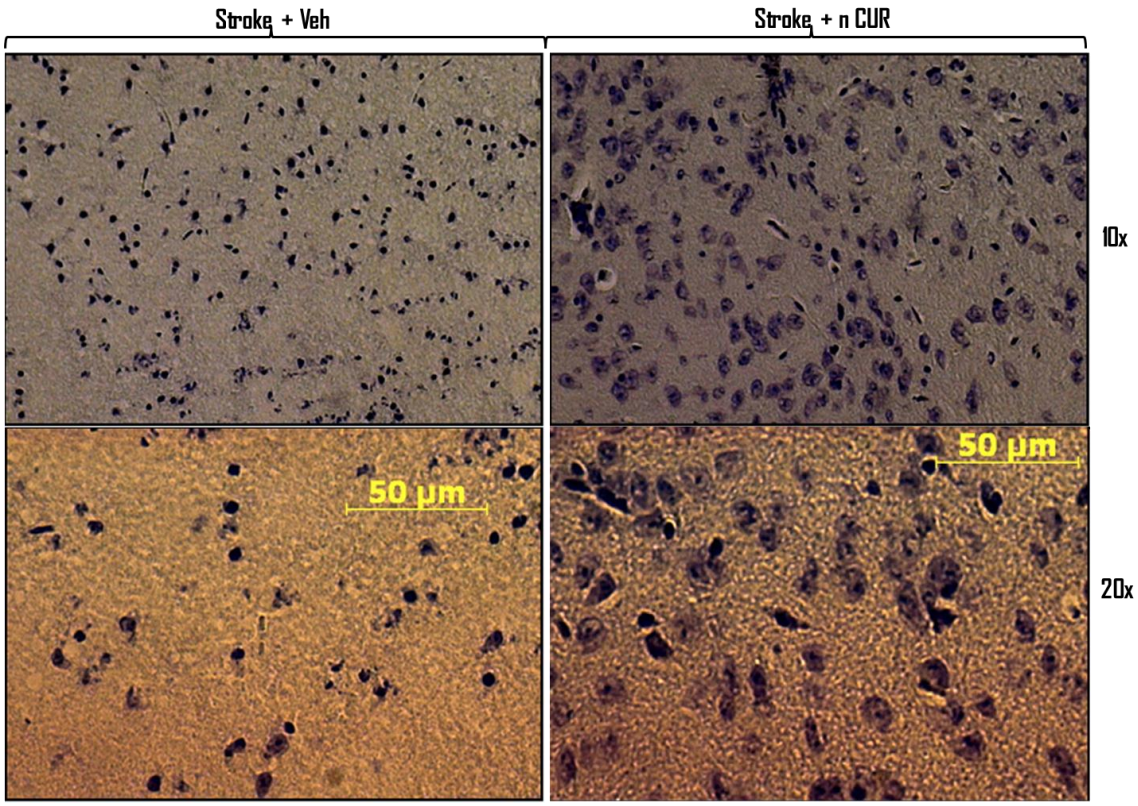

Figure 4.7 Haematoxylin and eosin stained brain section. The sections shows neuronal damage in the vehicle treated group compared to the nCUR treated.

4.4.3 nCUR inhibited the inflammatory gene expression

Inflammation is the hallmark of a majority of the acute neurovascular incidents including stroke (Lindsberg & Grau, 2003; Emsley *et al*., 2008). Therefore, CUR by virtue of its anti-inflammatory activity is believed to be effective in inflammatory mediated conditions (Ovbiagele, 2008). Therefore, we tested if nCUR holds this property after encapsulation in PLGA nanoparticles and serve as an affective anti-inflammatory agent after stroke. The mRNA expression of IL-1β, IL-6, inducible NOS (iNOS or NOS-2) and TNF-α were quantified. Acutely increased cytokines further aggravates cerebral microvascular dysfunction and hemorrhage. Therfore, all such factors which are possibly associated with the hemorrhage and limitation of therapeutic window of IV-tPA to 3 h, have been done at 6 hrs.

As evident from Fig 4.8a – Fig 4.8d inducing stroke increased the inflammatory gene expression significantly 6 h post stroke ($p \leq 0.01$ & 0.001 for Fig 4.8a & 4.8d and Fig 4.8b & 4.8d respectively). The nCUR treatment significantly reduced the gene expression of IL-1β ($p \leq 0.05$), IL-6 ($p \leq 0.01$) and iNOS ($p \leq 0.05$) while the effect was not significant on TNF-α expression as compared to the Stroke+Veh group, most likely because of inter-individual variability.

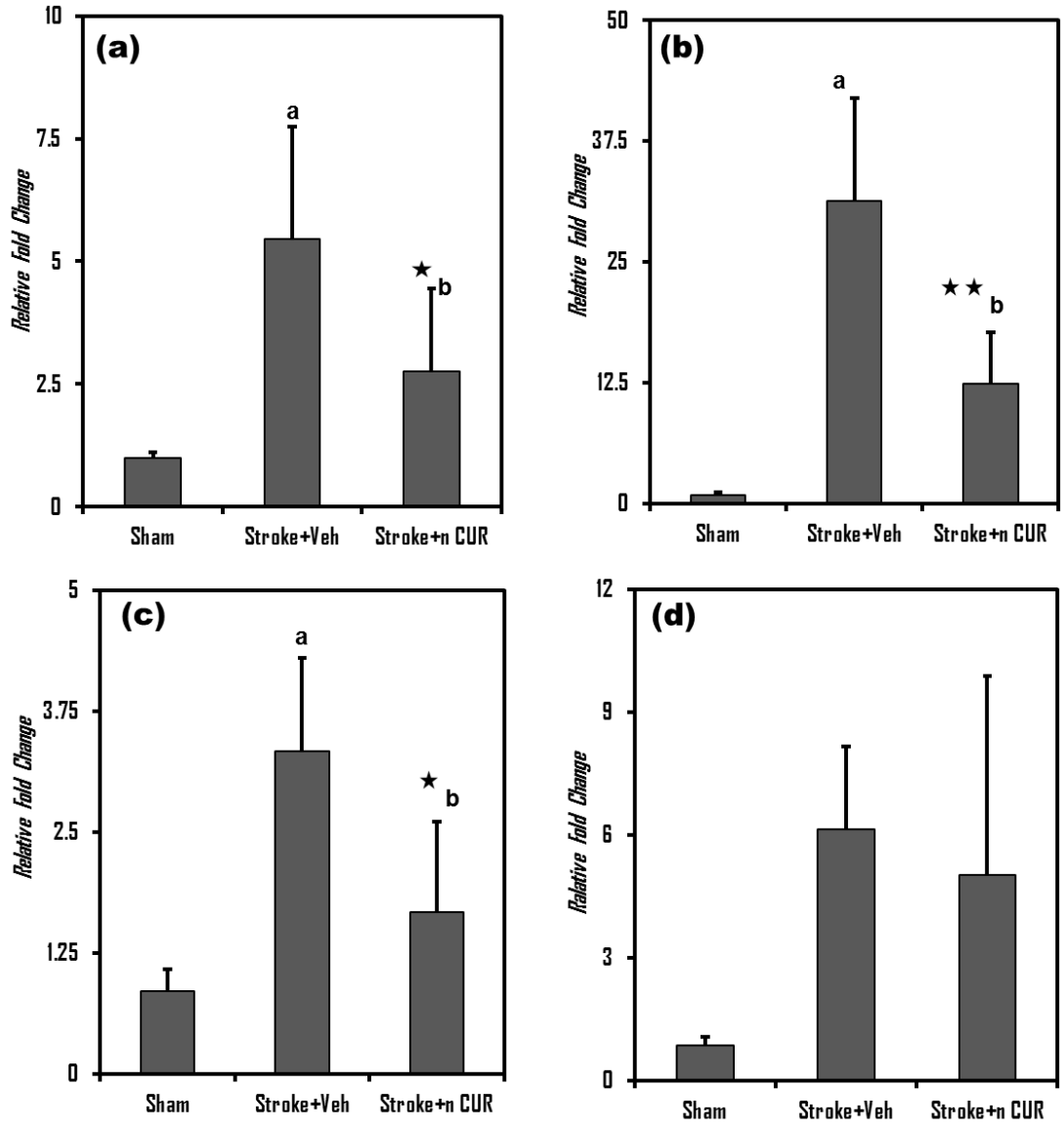

Figure 4.8 Inflammatory gene expression of **(a)** IL-1β, **(b)** IL-6, **(c)** inducible NOS (iNOS or NOS-2) and **(d)** TNF-α. Significant reduction of IL-, IL-6 and iNOS with nCUR treatment compared to the Stroke+Veh group (n=6). All values represent mean ± sd of the animals in a given group. *p<0.05 a vs b. **p<0.01 a vs b.

4.4.4 nCUR treatment reduced the post-stroke plasma peroxynitrite level, BBB leakage and edema formation

Stroke leads to oxidative-nitrative stress, which may contribute to increase the plasma peroxynitrite as a consequence of increased superoxide production (Sim *et al*., 2000). We found that eMCAO resulted in increased peroxynitrite level (Fig 4.9a) in the plasma as compared to the sham-operated group ($p \leq 0.001$). nCUR treatment attenuated the level of plasma peroxynitrite as compared to the Stroke+Veh group ($p \leq 0.01$). Increased peroxynitrite level may increase BBB leakage and edema formation. Therefore, we investigated the effect of nCUR treatment on post-stroke BBB leakage and infiltration. Plasma peroxynitrite is a potent activator of MMPs, and their time course of appearance correlates during hemorrhage. Hence we measured the levels of plasma peroxynitrite at 6 hr post stroke.

We found that the BBB leakage was significantly increased in Stroke+Veh group as compared to the sham operated mice at 24 h post-stroke (Fig 4.9b and 4.9c; $p \leq 0.001$) and it was reduced in the Stroke+N-CUR group as compared to the vehicle treated group ($p \leq 0.01$). Stroke+Veh group also had increased water retention (edema) (Fig 4.9d) as compared to the sham-operated group (83.1% vs. 76.7%; $p \leq 0.01$). nCur treatment significantly attenuated the edema formation after stroke as compared to the vehicle treatment (78.1 vs. 83.1; $p \leq 0.05$) at 24 h post-stroke.

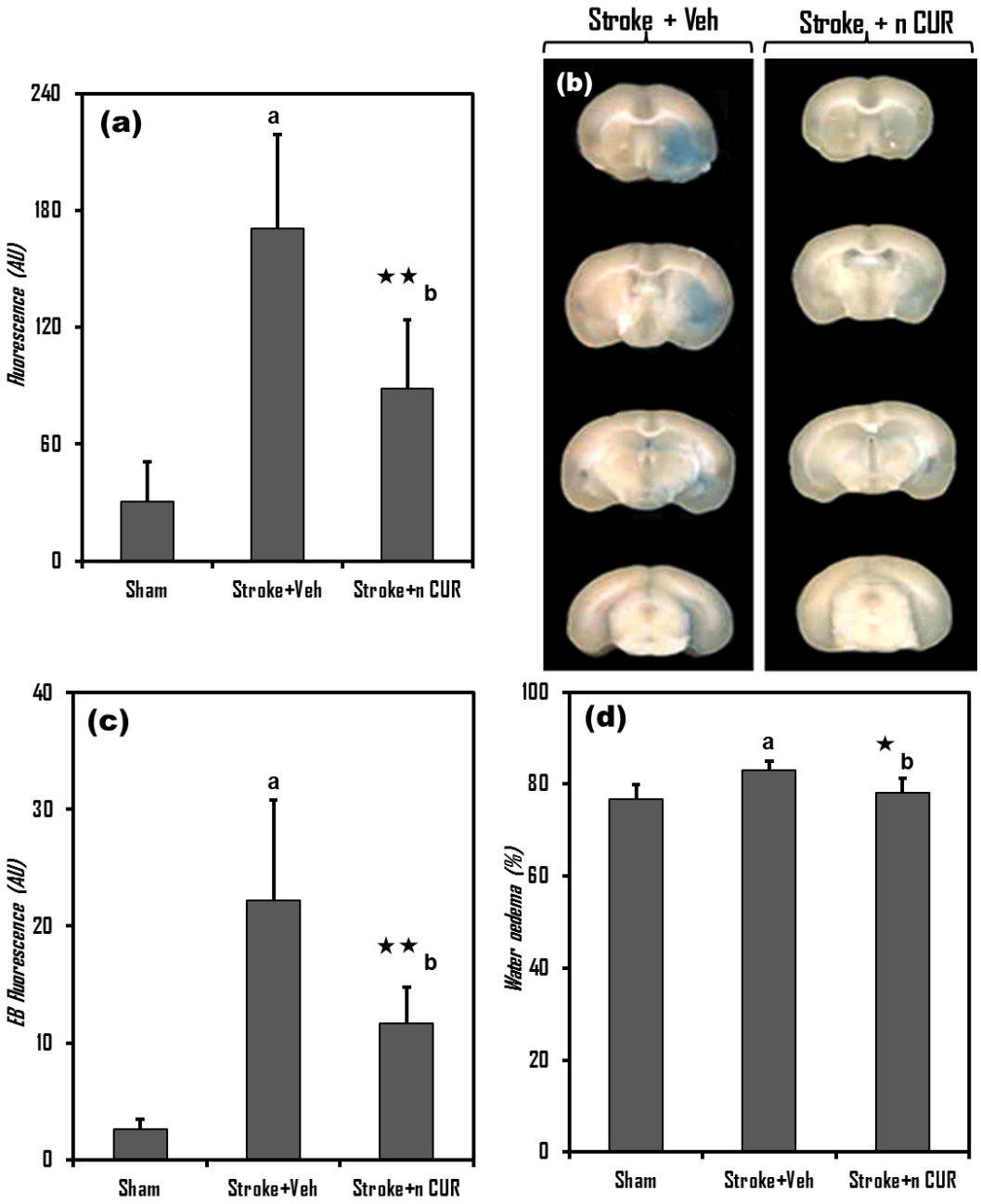

Figure 4.9 (a) Plasma levels of peroxynitrite at 6h post stroke significantly decreased by nCUR treatment. **(b)** & **(C)** Evans blue extravastion through BBB **(d)** Brain water content estimated after 24h post stroke. All values represent mean ± sd of the animals in a given group (n=6). *p<0.05 a vs b. **p<0.01 a vs b.

4.4.5 nCUR treatment attenuated the post-stroke MMPs expression and activity, a major cause of hemorrhagic transformation

Increased MMPs expression and activity is the major cause of hemorrhagic transformation after ischemic stroke and limits the therapeutic window of IV-tPA therapy. It additionally contributes into the BBB disruption and edema formation. Therefore, we next tested the potential of nCUR treatment in reducing the MMPs expression and activity in acute phase of stroke (6 h post-stroke). As detected by western blot analysis, stroke significantly upregulated the protein expression of inducible pro- and activated- MMP-9 as compared to the sham (Fig 4.10a and Fig 4.10b; $p \leq 0.05$). MMP-2, a constitutive MMP, was also upregulated but the change was not significant Fig 4.10c. nCUR treatment significantly reduced the protein expression of MMP-9 ($p \leq 0.05$) but the decrease in the protein expression of MMP-2 was not significant as compared to the Stroke+Veh group. The expression and activity of MMPs reaches to their peak at 6 hr post-stroke. Therefore, we looked for MMPs at 6 hr post-stroke. MMPs are highly responsible for post-stroke hemorrhagic transformation which limits the use of IV-tPA within 3 hr pots-stroke.

We also visualized the activity of these MMPs by *in situ* zymography (Fig 4.11). As indicated by the representative images, sham operated animal showed negligible MMPs activity (green fluorescence) possibly due to surgical procedure. In comparison, Stroke+Veh group clearly showed very high fluorescence, which was decreased after treatment with nCUR.

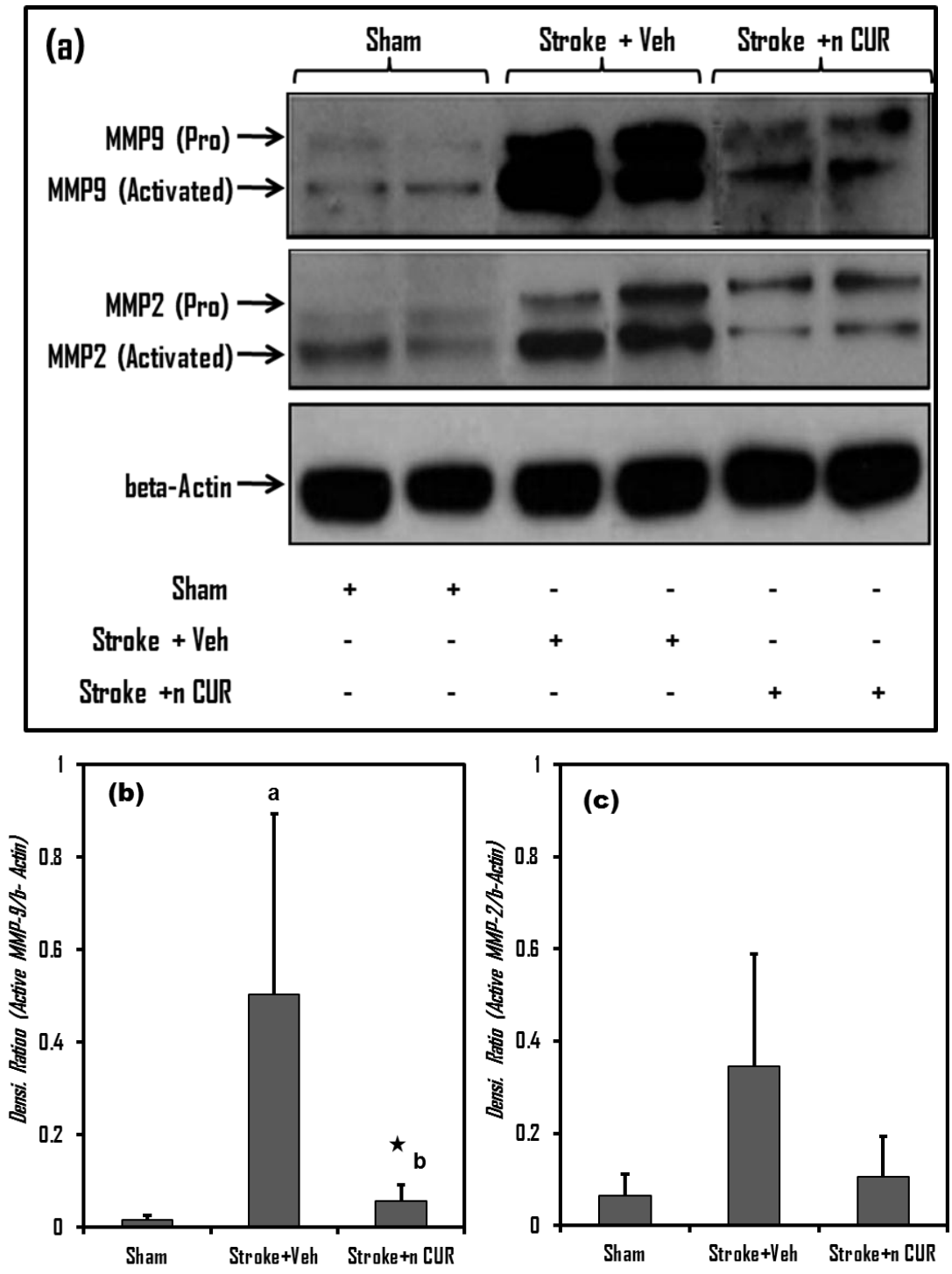

Figure 4.10 (a) Western blot analysis of pro and actin MMP9 and MMP2, **(b)** Pro and activated MMP9 and **(c)** Pro- and activated MMP2 expression. All values represent mean ± sd of the animals in a given group (n=6). *p<0.05 a vs b.

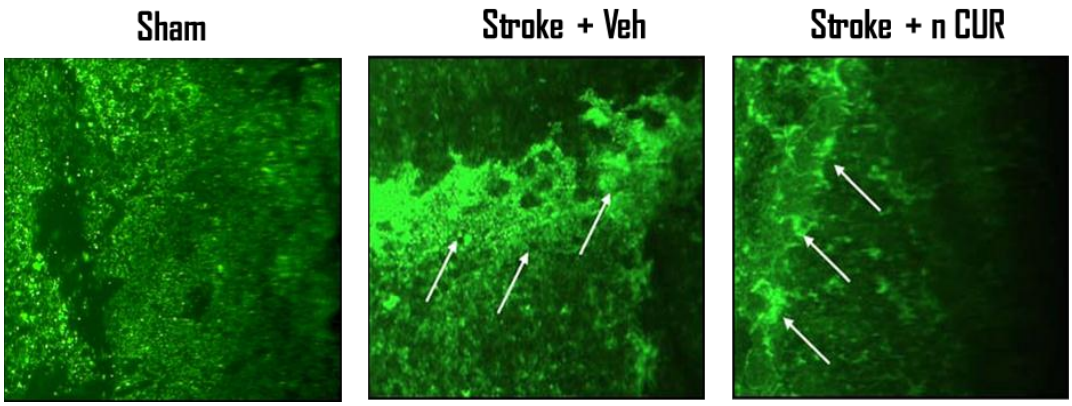

Figure 4.11 Zymographic images showing MMP activity represented as high green fluorescence activity**.**

4.4.6 nCUR treatment improves post-stroke survival

Edema is one of the reasons of increased mortality after stroke. Therefore, we finally evaluated the survival benefits after nCUR treatment post-stroke. As depicted in Fig 4.12 nCUR provides survival benefits after stroke. There was no mortality in sham-operated group mice while 4 out of 10 survived in the Stroke+Veh treated group, while 6 out of 10 mice survived in nCUR group indicating a survival rate of 33%.

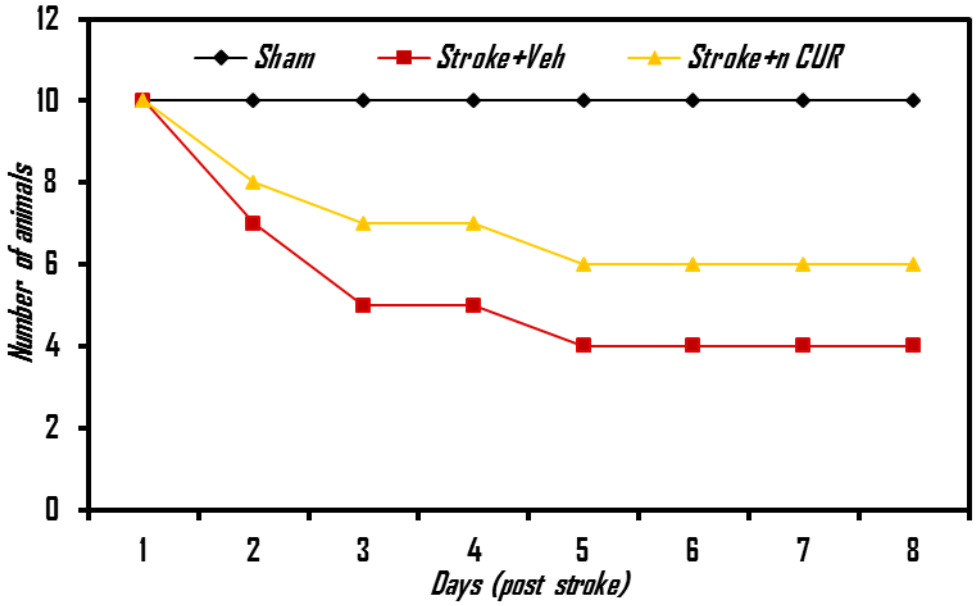

Figure 4.12 Post stroke survival rate of mice compared among different groups (n=10).

4.5 Conclusion

- The study proves that low dose (10 mg/kg) nCUR bears potential for neurovascular protection after embolic stroke compared to those used in the literature [plain curcumin 100-300 mg/kg] (Dohare *et al.,* 2008), probably due to enhanced bioavailability of the nCUR (Shaikh *et al.,* 2009).
- The nCUR improved post stroke survival rate by about 33% compared to the vehicle treated mice and this could be due to its ability to prevent oxidative stress, inflammation, MMPs over expression, BBB disruption and subsequent cell death.

5 EVALUATION OF nCUR IN MOUSE MODELS OF CANCER XENOGRAFT

5.1 Introduction

The uncontrolled and uncoordinated growth of the normal cells independent of the physiologic growth-regulatory stimuli is called neoplasia or tumour (Kumar *et al.,* 2007). Depending on its clinical behaviour, a tumour can be classified as either benign or malignant. Several factors such as geographical (life styles, occupation) age and genetics play a role in the pathogenesis of cancer (Kumar *et al.,* 2007; CRUK). The American Cancer Society (ACS) used the jointpoint method with modified Bayesian information criterion model to estimate the number of cancer deaths in 2012 (Chen *et al.,* 2012; ACS). Using the same methodology along with the WHO database, the number of deaths due to cancers in the EU was analysed and showed a positive drift except for lung and pancreatic cancers in women (Malvezzi *et al.,* 2013).

5.1.1 Ovarian cancer

Cancer of the ovaries is the most common gynaecological tumour (Ahmedin *et al.,* 2010) and accounts to the fifth and fourth most common cause of death in women worldwide and UK respectively (Auersperg, 2013; CRUK). The incidence of ovarian cancer was calculated using the Globocan Data (Ferlay *et al.,* 2010) and concluded the highest incidence is among the non-Hispanic White women and was followed by the Hispanic, African and Asian women. Genetic factors play a major role as a risk in developing ovarian cancers (Bell et *al.,* 2011; Koboldt *et al.,* 2012; Rafnar *et al.,* 2011). The risk of developing ovarian cancer is about three or four times increased in women having

history of cancer in the first degree relative (Granström *et al.,* 2008). Reproductive factors such as parity (Granström *et al.,* 2008), breast feeding (Danforth *et al.,* 2007), infertility (Tworoger *et al.,* 2007), use of exogenous hormones (Beral *et al.,* 2007) have shown to be risk factors associated with ovarian cancer. Evidence indicates that lifestyles factors (e.g. smoking, physical activity, diet) are mixed and inconclusive (Schulz *et al.,* 2005; Genkinger *et al.,* 2006; Rossing *et al.,* 2010). Other medical conditions associated with risk of pelvic inflammatory conditions such as endometriosis, ovarian cysts and Lynch syndrome have also been associated increased risk of developing ovarian cancer (CRUK).

The current approaches in early detection of cancer are multiple tumour marker and transvaginal ultrasound. Recently, number of the markers have been identified and investigated that show promising results in the early detection of ovarian cancer. CA-125, Human Epididymis Protein 4 (HE4), Mesothelin, human kallikrein (KLK6 and 7) genes, Glutathione S-transferase 1(GSTT1), Human prostasin (PRSS8), folate receptor alpha (FOLR1), Aldehyde Dehydrogenase 1(ALDH1) and microRNAs (miRNAs) are some of the markers that could significantly improve the overall survival rate of ovarian cancer patients (Sarojini *et al.,* 2012).

The management plan of ovarian cancer depends on the stage of ovarian cancer. Most of the ovarian cancers are diagnosed in its late stage and combination of surgical and platinum based chemotherapy is the standard treatment. In the last three decades, novel treatment strategies targeting at multiple molecular pathways have been identified (Kim *et al.,* 2012). Bevacizumab (Avastin), a recombinant humanized monoclonal IgG1 anti-VEGF monoclonal antibody is the most promising molecular drug exhibiting its anti-tumour activity by binding with VEGF ligands. Several phase trials

(GOG 218, ICON7, OCTAVIA, OCEAN and JGOG) have shown promising results both as in the first-line, recurrent treatment of EOC (Perren *et al.,* 2011; Burger *et al.,* 2011). Olaparib which is a PARP inhibitor acts by repairing the breaks in the single stranded DNA has shown to improve the prolonged the median progression-free survival (PFS) when administered to subjects with platinum-sensitive, recurrent, high-grade serous ovarian cancer (HGS-OC) (Ledermann *et al.,* 2012).

5.1.1.1 Ovarian cancer model

To evaluate the efficacy of the novel therapeutic formulations, it is critical to develop animal models so it mimics the behaviour of ovarian cancer in human subjects. Earlier, induction of ovarian cancer was reported using chemical such as 7, 12- Dimethylbenz (a) anthracene (DMAB) (Toth 1970) but DMAB induced neoplastic changes in endometrium, cervix as well as fallopian tube. N-methyl-N'-nitrosourea and 20-methylcholanthrene, 1, 3-butadiene, formic acid 2- (4-(5-nitro-2-furyl)-2-thiazolyl) hydrazide (Tunca *et al.,* 1985) are other chemicals used as carcinogens to induce ovarian tumourigenesis. In the last decade, major advances have been made. The mouse ovarian surface epithelial (MOSE) cells were isolated and cultured *in vitro* to develop syngeneic ovarian epithelial tumour models (Roby *et al.,* 2000). These cells spontaneously transformed to tumour phenotypes and were limited to only few numbers of animals as it was not an inherited trait. The genetically engineered models (GEM) have provided valuable information and have been successful differentiating high grade serous adenocarcinoma (Szabova *et al.,* 2012) and low grade serous adenocarcinoma (Fan *et al.,* 2009). Among GEM, the transgenic models are considered to give

consistent and predictable results. These mouse models exhibit the various subtypes of cancer exhibited in the women and provide evidence of the oncogenic factor responsible in ovarian surface epithelium. It has also provided information about cell signalling and expression pathways for management of cancer.

However, currently subcutaneous and intraperitoneal xenograft or transplant into immunosuppressed mice is broadly accepted model. The transplant material is obtained directly from the human ovarian tissues that could match the original tumours.

5.1.2 Lung cancer

According to the statistics by cancer research, currently the most frequent cancer in the world is lung cancer and has been in this trend for several decades (Parkin *et al.,* 2001; Ferlay *et al.,* 2010). In the UK, lung cancer is the second most common cancer diagnosed after breast cancer and prostate cancer in women and men respectively (CRUK). It is also considered to be the most common cause of death worldwide and in UK (ACS; CRUK; Siegel *et al.,* 2012). The incidence rate of lung cancer is more than twice in men compared to women (Ferlay *et al.,* 2010). Smoking is considered to be the predominant risk factor (WCRF/AICR) and correspondingly increases the risk with the duration and quantity of smoking (ACS). Environmental exposure to gases (Radon), smoke, metals (asbestos, chromium, cadmium and arsenic), radiations and paints are other culprits associated with lung cancer (ACS; CRUK). Occupations such as chimney sweeping, paving, roofing and rubber manufacturing are also considered as other additional factors associated with risk of lung cancer (Brüske-Hohlfeld 2009). Several

studies and data currently remain conflicting and unclear of the role of human papilloma virus (HPV) as a cause of lung cancer (Syrjanen *et al.,* 2012). On the basis of the results summarized by the meta-analysis, the role of genes as a contributing factor in the development of lung cancer could not be defined as the study was limited to individual ethnic groups, and a small study size was used (Marshall & Christiani 2013).

Earlier, annual chest radiograph and cytological analysis of sputum were the used as screening methods for early detection in high risks patients but without much success (Oken *et al.,* 2011). National Lung Screening Trial showed decreased mortality among the high risk group of subjects by 20% screened by low-dose spiral computed tomography scan compared to those screened by chest x-ray (Aberle *et al.*, 2011) but is associated with its own limitations and risks (Jett, 2012). Recently, a number of advances have been made at molecular level in gene expression, protein expression and metabolic activity in tissues and biofluids for early identification of subjects who are at increased risk of developing lung cancer (Hassanein *et al.,* 2012).

The management of lung cancer depends on the stage, type and position of cancer and includes surgery, chemotherapy and radiotherapy on its own or in combination. Clinical trials with small molecules such as Erlotinib (Tarceva), and gefitinib as first line for advanced non-small cell lung cancers showed a high response rate and longer free survival rates when compared to those who received chemotherapy (Mok *et al.,* 2009; Rosell *et al.,* 2012). Crizinotib (Xalkori), another small molecule which is an ALK- tyrosine kinsae inhibitor (TKI) had a dramatic response on patients and currently phase III trials are in progress (Soda *et al.,* 2007; Kwak *et al.,* 2010). Bevacizumab (Avastin), an anti-vascular endothelial growth factor

neuroclonal antibody in combination with paclitaxel/carboplatin was gave superior results (Scagliotti *et al.,* 2008).

5.1.2.1 Lung cancer model

About 80% of the lung cancers are non-small-cell lung carcinoma (NSCLC) and they differ significantly histopathologically from small-cell lung carcinoma (SCLC) and hence differ in the treatment (Meuwissen & Berns, 2005). Therefore, to meet these requirements a variety of lung tumour models have been developed that offers help in drug screening (Meuwissen & Berns, 2005). The transgenic models resemble the lung tumour models in human and thus providing us with the knowledge the roles of oncogenes in the initiation and development of lung cancer (Johnson *et al.,* 2001; Meuwissen & Berns, 2005). Despite the increased sophistication of the models developed, most of the research activity today is conducted on tumour models which are less clinically accurate. Recently, the patient derived-xenograft models (PDX) have made a major comeback as a promising model for studies (Moro *et al.,* 2012).

In this study we used B16 F0, a cell line derived from melanoma which has high metastatic capacity, is widely used and is the best characterized model (Fidler & Nicolson, 1977). The advantages of this model are its reproducibility and rapidity at which the model developed. The potential disadvantage of this model was the cascade in the disease progression could not be followed. A Bioluminescent Imaging (BLI) tool was used to quantitate the growth of cancer noninvasively. It serves as a rapid monitoring of the tumour progressing offering insights into early events in tumour development or lead to shorter experimental procedures without having to

kill the animal (Lim *et al.,* 2009) by injecting luciferin where the tumours radiate a visual light signal that can be assessed using a sensitive optical imaging system such as the IVIS Spectrum in the present case. The region of interest around the tumour sites was subratcted against the background bioluminescence and quantified as total proton counts using the Living image software.

The quantum of light from the tumour is comparative to the number of light emitting cells and this signal can be measured to monitor tumour growth and development. The instrument is calibrated to enable absolute quantitation of the bioluminescent signals. A series of events can lead to reduction in the bioluminescent signal such as tumour cell death and necrosis due to decreased oxygen supply or drug treatment. However, the cell death might not always be accompanied by a reduction in tumour size as measured by physical means.

5.2 Specific aims

- To assess the efficacy of orally delivered nCUR in ovarian and lung cancer models.
- To understand the target tissue levels of curcumin in an attempt to correlate between bioavailability and therapeutic efficacy.

5.3 Materials and Methods

5.3.1 Materials

The human ovarian carcinoma cells, A2780 were obtained from Dr. T. C. Hamilton (Fox Chase Center, Philadelphia, USA). The murine melanoma cell lines B16 F0 luciferase (B16 F0 luc) and luciferin were obtained from Caliper

Life Science Ltd. (Runcorn, UK). Dulbecco's Modified Eagle Medium (DMEM) was purchased from Invitrogen (Paisley, UK). Rest of the materials were procured as described in the previous chapters.

5.3.2 Preparation and characterization of Ncur

The nCUR were prepared as mentioned in section 2.3.2/2.35 and characterization performed as described in the section 2.3.6.

5.3.3 Ovarian cancer model

5.3.3.1 Cell culture

The human ovarian carcinoma cell lines, A2780 is sensitive to many cytotoxic drugs such as paclitaxel and doxorubicin. They were grown in a Roswell Park Memorial Institute 1640 (RPMI 1640) medium containing glutamine (2 mM) and foetal calf serum (10%).

Cells were grown in 75 cm^2 T-flasks to obtain ~90% confluence and the culture medium was changed every alternate days. The temperature conditions required for culturing these cells were of ~37 °C in an atmosphere of ~5% CO^2 and ~85% relative humidity.

5.3.3.2 Efficacy study design in vivo

All the animal studies adhered to the UKCCR guidelines for welfare of animals and conducted under the project licence issued under U.K. Home Office Animals (Scientific Procedures) Act 1986. This study was conducted at The Beatson Institute, Glasgow under the guidance of Dr Jane Plumb.

Female nude mice aged 6-8 weeks were used in the study. The drug sensitive 2780 cells grown in cell culture flasks were harvested by exposure to trypsin-EDTA, washed three times in PBS. The detached cells were isolated in equal volume of culture medium and centrifuged at 300g for 5 min. The pellets were re-suspended in PBS. 5×10^6 cells in 0.1 ml was injected subcutaneously into the right flank of athymic nude mice (CD1 nu/nu mice). The tumour took approximately 7-10 days to reach a mean diameter of 0.5 cm or more and then the animals were randomized into 6 groups (n = 6). Group 1 did not receive any treatment, Group 2 received 100mg/kg CUR suspended in CMC and administered orally in a volume of 10µl/g body weight, Group 3 received CUR IP at 100mg/kg in 10 µl/g, Group 4 received nBLA (equivalent to 100 mg/kg CUR) by oral route, Group 5 and Group 6 received nCUR by oral and IP routes at 100mg/kg equivalent CUR in a volume of 10µl/g body weight. The animals were treated on day 0, 2, 4, 6, and 8. The mice were weighed daily and tumour volume was measured and calculated using a caliper assuming spherical geometry formula.

The results are presented as the relative tumour volume and body weight (mean ± SEM of 5 mice) in which for each mouse the measurements are expressed relative to that on the first day of treatment (Day 0).

5.3.4 Lung cancer model

5.3.4.1 Cell culture

B16 F0 luc cells were grown in a DMEM (Dulbecco's Modified Eagle Medium) containing 5 mg/ml streptomycin, 2000 mM LGlutamine and 5,000 U/ml penicillin in a 75 cm^2 T-flasks supplemented with 10% foetal bovine

serum (FBS) which was heat- inactivated and passaged every 24 or 49 h to obtain ~90% confluence.

5.3.4.2 Tissue distribution study

The tissue distribution experiments were conducted at University of Strathclyde on mice weighing 20-30 g. After one week of acclimatization, mice were randomly and equally divided into three groups (n=27): Group1 received 100 mg/kg CUR in CMC; Group 2 received 500 mg/kg CUR in CMC and Group 3 received 100 mg/kg nCUR. All animals received single dose and were administered by oral route. Three animals from each group were sacrificed at 2 h, 12 h and 24 h. Blood was collected by heart puncture in heparinised tube and plasma was separated by centrifuging the blood at 3000 rpm for 10 min at 4°C. Lung and liver were isolated and stored in -80 till further analysis.

The tissues samples (plasma, liver and lung) were analysed for curcumin using a validated *in vivo* HPLC method (Shaikh *et al.*, 2009). In brief, to 250 µl of plasma, 25 µl of 2.8% of acetic acid and 50 µl of 17β-estradiol acetate as IS was added and vortexed for 20 s. 1.2 ml of ethyl acetate was used for extraction followed by voterx for 15 min and later centrifuged at 10,000 rpm for 15 min at 4°C. The organic layer was collected and evaporated to remove ethyl acetate. The residue was reconstituted in 130 µl of methanol and analysed using *in vivo* HPLC method. The tissue samples (1 g) were homogenised in 7.4 pH ml of 4 ml phosphate buffer and followed the same procedure of plasma extraction for tissue extraction using 250 µl of sample.

5.3.4.3 Efficacy study design in vivo

Male BALB/c mice six to eight weeks weighing 20-30g were injected with 5 × 10^5 B16 F0 luc cells in 0.2 ml of RPMI into tail veins of the mice. All studies were approved and carried out under a Project Licence issued under the U.K. Home Office Animals (Scientific Procedures) Act 1986 at University of Strathclyde under the guidance of Dr Carter. The animals were housed under conditions of controlled illumination (12:12 h light/dark cycle), humidity, and temperature (18–22°C). Tumour progression was monitored in mice using IVIS imaging of mice injected intraperitonally with 150 mg/ml luciferin solution (16.89 mg/ml PBS pH 7.4). During imaging, isofluorane anesthesia was maintained using a nose cone delivery system and the body temperature of the animals was maintained using a digitally thermostated bed integrated within the IVIS system. Mice were imaged 10 min after injection and the total flux (p/s) for the lung area measured. The animals were then divided into 2 groups. Group 1 (n = 4) were treated with PBS and the Group 2 received five doses of nCUR at 100 mg/kg body weight by oral route each day on days 3-7. The mice were euthanized on day 11 and the lungs were grossly inspected and weighted.

Statistics: The organ weight, tumour parameters and tumour effect of drug treatment on cell proliferation in *in vivo* drug studies were analysed using Mann Whitney U test. A p value of < 0.05 was considered significant.

5.4 Results and discussion

5.4.1 Preparation and characterisation of nCUR

The Z-average particle size and PDI of nCUR obtained was 290.6 ± 1.29 nm and PD1 of 0.08 ± 0.01 with curcumin entrapment of approximately 56%. The size of nBLA was 254.2 ± 2.12 nm and 0.17 ± 0.01 PDI.

5.4.2 Ovarian cancer model

Contradictory to the literature reports (Zheng *et al.,* 2006; Lin *et al.,* 2007) curcumin failed to halt the tumour growth in A2780 xenograft (Fig 5.1). The body weights of the mice also remained unaltered in all the groups (Fig 5.2). Recently, in one of the reports curcumin encapsulated PLGA nanoparticles demonstrated superior anticancer potential in cisplatin resistant A2780CP ovarian and metastatic MDA-MB-231 breast cancer cells (Yallapu *et al.,* 2010) in which nanoparticles were effective at 9-14 μmol concentration while the plain curcumin is effective at slightly higher concentration ~16 μmol that translates to approximately 3.3 mg to 5.8 mg, though these doses used in cell studies cannot be easily extrapolated to animal doses, nevertheless could high concentrations for cell assay. The doses used in the present study are about 2.5 mg/mice which is much lesser than that was used in the cell studies. The doses that lead to success *in vivo* are much higher than that we tested in this study. Aggarwal and colleagues conducted a dose finding study in which they reported concentrations beyond 500 mg/kg that is five times higher than the dose we used in the current study, will have ability to inhibit the NF kB activation that is responsible for suppressing inflammation and angiogenesis (Lin *et al.,* 2007). A wider variety of human ovarian cell lines were used to produce xenograft models for testing anticancer agents,

however and these models are reported to have varied features in ploidy, doubling times and clonogenicity (Wilson *et al.*, 1996) which can render different activity for the similar treatment modalities. From the formulation viewpoint it is not feasible to administer 500 mg/kg doses using nanoparticles or going beyond 100 mg/kg also seems to be a tedious task considering the volume of sample we can administer to mice, for nanoparticles of ~56% curcumin encapsulation at 15% initial drug loading. The scope for dose escalation exists if we can improve the initial loading and entrapment efficiency. Though the initial studies from our laboratory demonstrate 9-fold improvement in oral bioavailability rats, we have not seen curcumin in the nCUR form being effective in the xenograft model though we don't understand the bioavailability of curcumin in the xenograft model though all the treatment forms show similar tumour burden that confirms curcumin is ineffective at this dose.

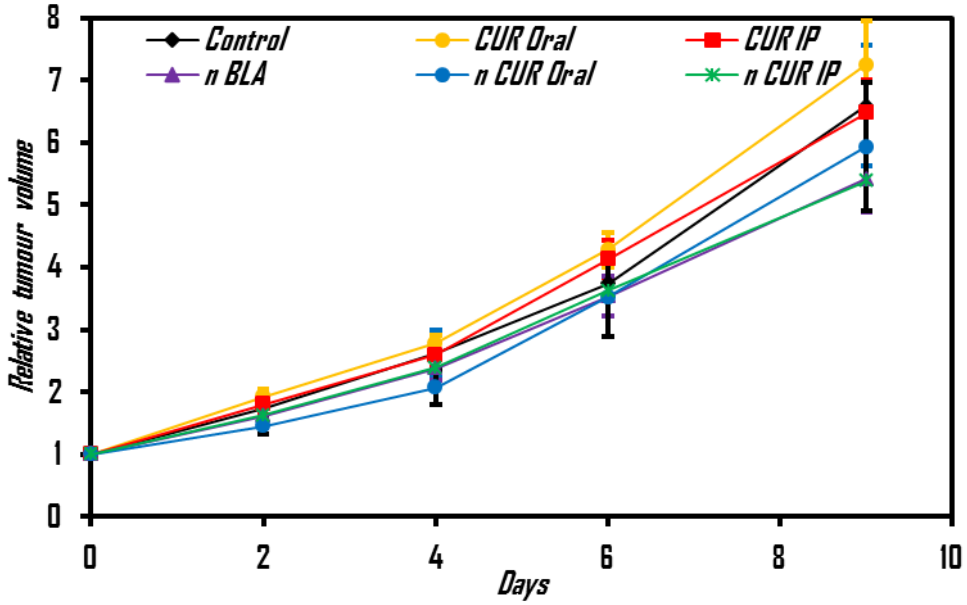

Figure 5.1 The effect of CUR and nCUR on tumour growth in A2780 xenograft. The mice were treated approximately after 10 days (after tumour

had grown to ~0.5 cm) on days 0, 2, 4, 6 and 8 by oral and IP routes. All values represent mean ± sd.

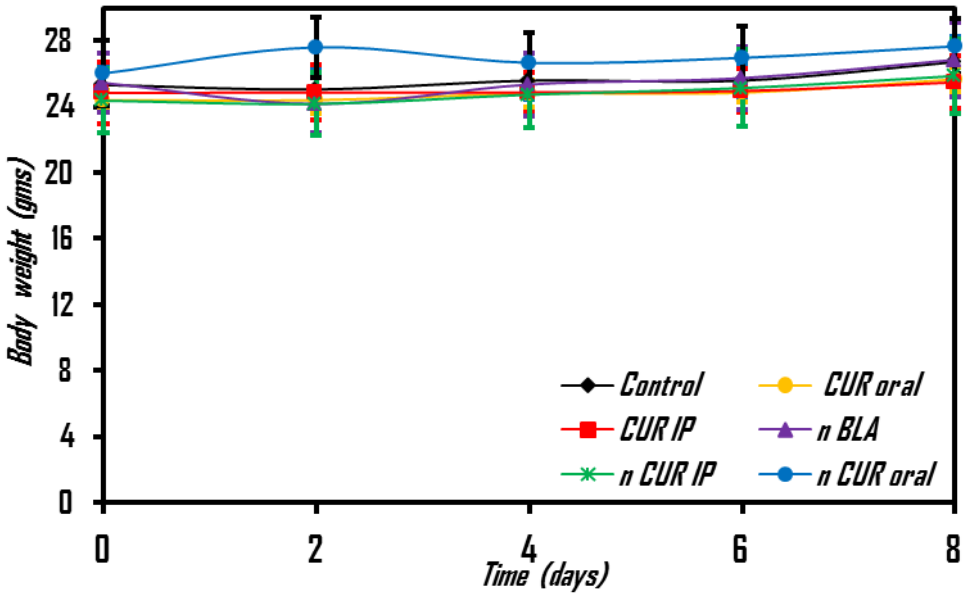

Figure 5.2 The relative body weight of mice undergoing different treatments expressed relative to that on day of treatment (day 0). All values represent mean ± sd.

5.4.3 Lung cancer model

5.4.3.1 Tissue distribution study

Having realised 100 mg/kg is ineffective in the ovarian cancer model and the difficulty in escalating the dose with current nCUR we performed this *in vivo* tissue distribution study in mice, where in CUR was administered 500 mg/kg and nCUR 100 mg/kg. The curcumin levels in lung at 2 h were comparable between 500 mg/kg CUR and 100 mg/kg nCUR while 100 mg/kg CUR shows much lower levels, and the difference between these groups seems to narrow down at 12 and 24 h, the concentrations on whole seems to decrease at 24 h (Fig 5.3). The levels of curcumin in lungs were significant ($p< 0.05$) with CUR 500 mg/kg and 100 mg/kg nCUR at 2h. The nCUR shows very high levels in

the liver compared to CUR while there is no difference between 100 and 500 mg/kg CUR. At 12 h all the 3 groups seem to be comparable while nCUR last until 24 h (Fig 5.4) Curcumin is known to induce phase I and phase II enzymes which in return are responsible for its metabolism in the liver. The initial higher levels of curcumin induces higher amount of the enzymes thereby higher metabolic rates, however with time this remains constant (Bansal *et al.,* 2011), at least for 12 and 24 of nCUR. Overall, this study demonstrates 100 mg/kg of nCUR results in better or comparable tissue levels of CUR in the tissues studied.

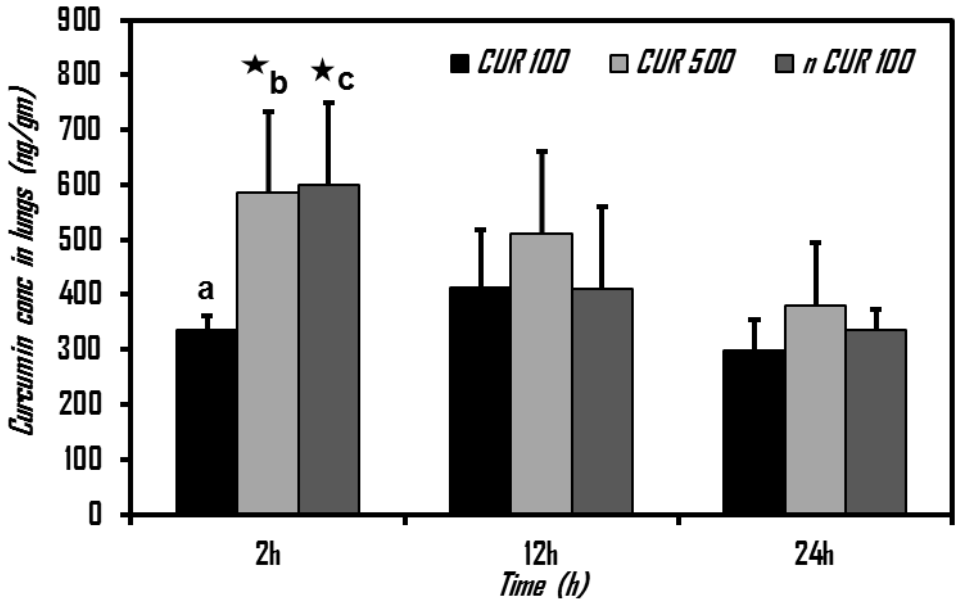

Figure 5.3 Tissue distribution of curcumin in lungs of mice (n=3) after 2 h, 12 h and 24 h of oral dosing. $p<0.05$ a vs b and a vs c. All values represent mean ± sd.

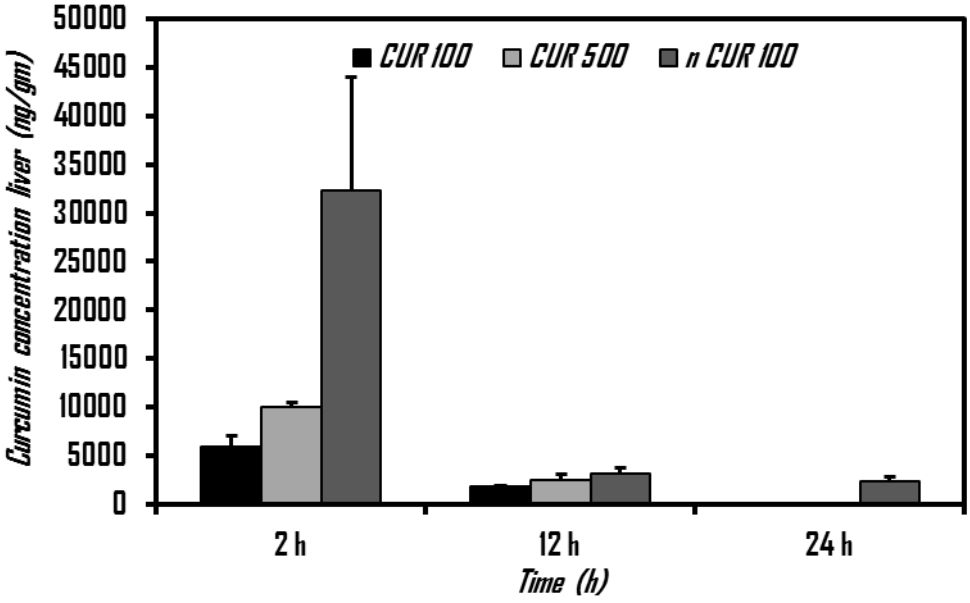

Figure 5.4 Tissue distribution of curcumin in liver of mice (n=3) after 2 h, 12 h and 24 h of oral dosing. All values represent mean ± sd**.**

5.4.3.2 In vivo cancer efficacy studies

The B16 F0 luc cell lines administered through I.V route invaded and colonised in the lungs as black patches. The mean weight of the BALB/c mice in both the groups showed no significant variation on body weight (Fig 5.5). The variation in the weights of lung and liver between the control and treated groups was insignificant (Fig 5.6). The implanted tumours in the lung did not respond to nCUR. Overall, from the studies it appears that 100 mg/kg nCUR irrespective of its ability to match 500 mg/kg CUR lung distribution failed to shrink tumour.

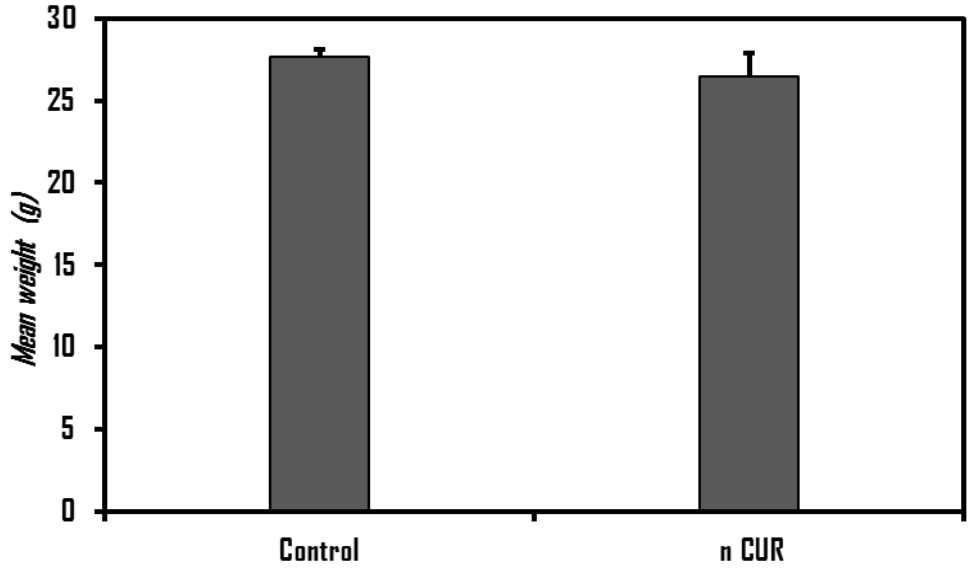

Figure 5.5 Mean weight of BALB/c mice. All values represent mean ± sd**.**

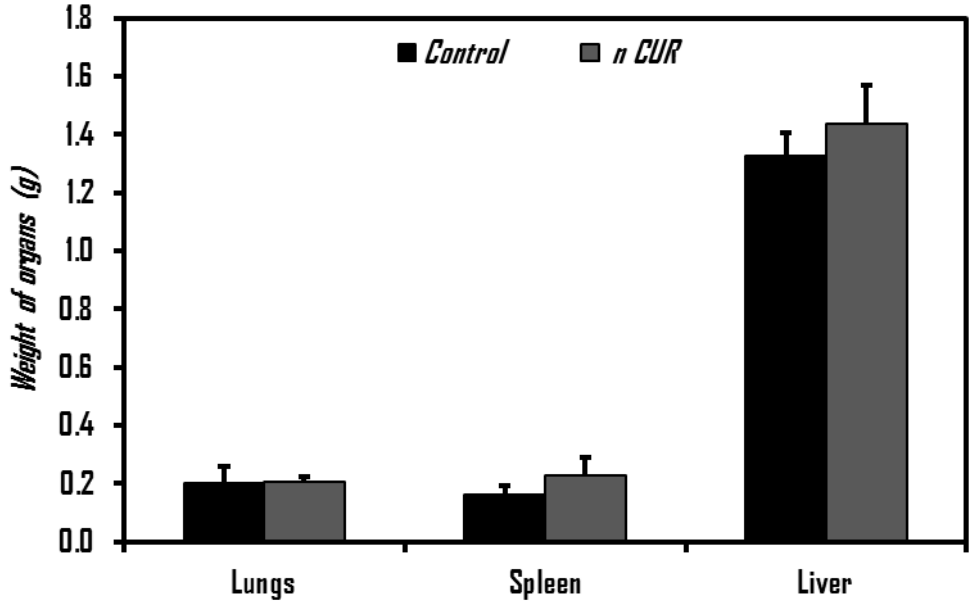

Figure 5.6 Weight of lungs, spleen and liver of the control and nCUR treated group. All values represent mean ± sd**.**

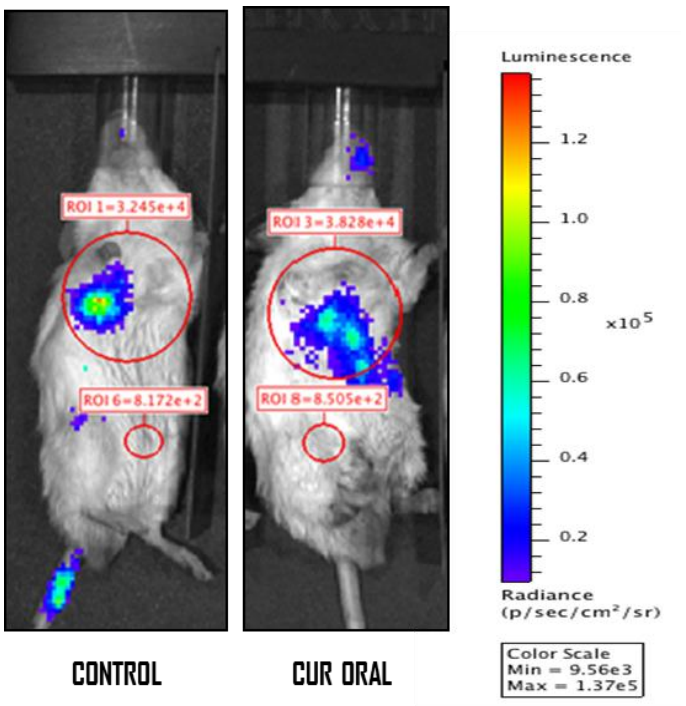

Figure 5.7 Bioluminescence images of mice on day 11 showing intratumoural luciferase activity. The scale to the right of the BL images describes the colour map for the photon count.

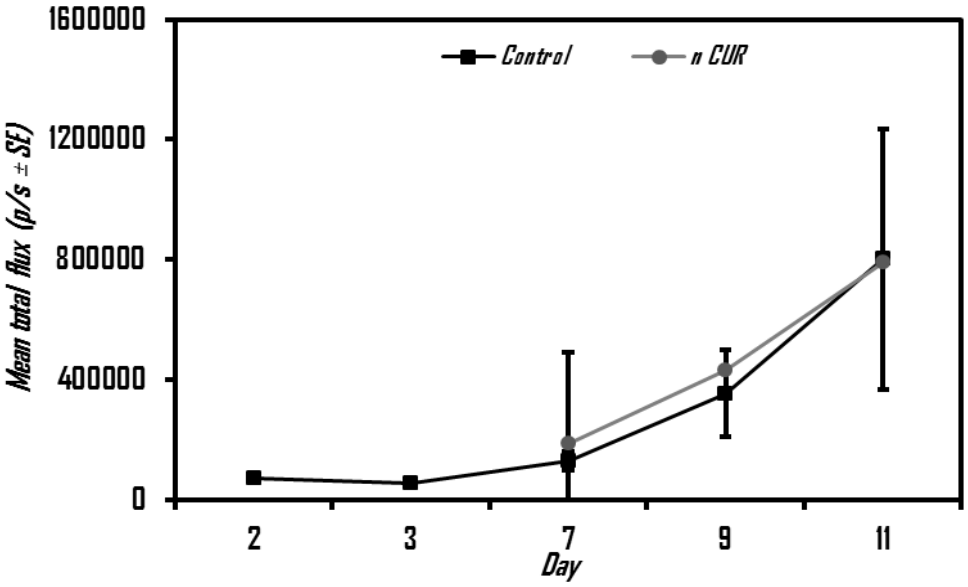

Figure 5.8 Tumour volume measured as total flux over 11 days. All values represent mean ± sd.

5.5 Conclusion

- Orally administered nCUR did not have any effect on the growth rate of the tumour in the xenograft models at the dose used in the study.
- nCUR were superior or comparable to CUR in the tissue distribution study at 5 times lower dose.

6 FUTURE DIRECTIONS

In the present dissertation a successful scale-up of nCUR and its therapeutic activity in diabetic cataract and stroke models was demonstrated, while nCUR was ineffective in cancer models in the studied conditions. Considering the initial success with nCUR the following future plan is suggested.

Appropriate ways to go beyond the pilot-scale production of nCUR should be consider wherein the use high pressure homogeniser (HPH) such as The Avestin (Avestin Inc., Ottawa, Canada) should be considered. Optimal centrifugation procedures at industrial scale should be considered or develop alternative methods such as ultra-filtration for removal of unbound materials from the preparation. It is essential to maximise the initial loading and the entrapment efficiency curcumin while keeping the particle characteristics such as size and PDI similar or reduce. For this purpose polymers in similar category with little high molecular weight or high crystalline component should be considered wherein higher entrapment could be possible due to high hydrophobicity of the polymers.

In the diabetic cataract study, the dose of CUR and nCUR used was 2 mg/day/rat and the results were promising. Hence, a dose response study with the most appropriate nCUR (considering the entrapment, size etc) should be considered. It is clear that the initiation and progress of diabetic complication is multi-factorial wherein combination therapy could prove beneficial. Adding another antioxidant to the nCUR such as CoQ10 that can target mitochondrial dysfunction known have significant role in diabetes and complications. For combination treatment two individual nCUR or nCOQ10 can be used or a co-encapsulated product can be developed. Similarly,

antioxidant combinations with insulin can prove beneficial too as insulin seems to be the most dependable treatment option for diabetics.

The data on stroke is certainly promising and nCUR is effective at as low as 10 mg/kg which is very low compared to the literature reports that used as high as CUR 300 mg/kg. So certainly there lies a room for dose response which need to be investigated. rtPA is the only approved vasculo-protective therapy till date. Due to high risk-to-benefit ratio, it only benefits 23% of the victims if delivered within 3-4 h post incidence. It will be interesting to investigate if nCUR as a combination to tPA therapy can increase the window of tPA, in different sexes and its effectiveness after stroke under comorbid conditions like diabetes and hypertension.

Finally, our data in cancer models was disappointing where nCUR was ineffective in both the models studied. However, in the ovarian cancer model it is interesting to note the treatment groups from day 8 onwards started showing some difference to that of untreated suggesting the an increase duration need to be considered. Nevertheless, curcumin alone for cancer doesn't seem to be a viable option considering the effect at such high doses. While, recent reports suggest curcumin could be a potential candidate to improve the drug sensitivity and overcome multidrug resistance of compounds such as paclitaxel (Ganta & Amiji, 2009; Ganta *et al.,* 2010). Therefore, attempts should be made to develop CUR-paclitaxel co-encapsulated nanoparticles.

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