CHAPTER V

EVALUATION OF CURCUMIN LOADED SOLID LIPID NANOPARTICLES IN ANIMAL MODEL OF ALZHEIMER’S DISEASE
1.0 INTRODUCTION

Alzheimer's disease (AD) is a progressive fatal neurodegenerative disorder involving chronic CNS inflammation leading to significant oxidative damage (Friedlich and Butcher, 1994; Montine et al., 1999; Smith et al., 1997). The foremost characteristic feature of the disease is a progressive cognitive deterioration (Standaert and Young, 2001) coupled with declining daily activities and behavioural changes. Marked atrophy of the cerebral cortex and loss of cortical neurons are the hallmarks of AD. The neuropathological investigations of AD, in human brain, illustrate amyloid rich senile plaques (Selkoe, 2000), neurofibrillary tangles (Spillantini and Goedert, 1998) and neuronal degeneration with impairment of short-term memory.

A comprehensive animal model imitating all the cognitive, behavioural, biochemical, and histopathological abnormalities observed in AD patients does not exist (Rui et al., 2008). Nevertheless, various reports on a partial reproduction of AD have been achieved with various pharmacological approaches such as cholinergic dysfunction-related and amyloid peptide related animal models, and transgenic animal models (Games et al., 1995; Pepeu et al., 1996; Yamada et al., 1990).

Various researchers report on the potential toxicity of aluminum in experimental animal models and in humans under different clinical conditions (Domingo, 1995; Garcia et al., 2009; Llobet et al., 1987; McLaughlin et al., 1962). Aluminium (Al) silicate granules have been observed in the brains of patients with AD (Candy et al., 1986), and the symptoms associated with aluminium chloride (AlCl₃) induced brain toxicity in animals (mice) fairly resemble those of AD (Rui et al., 2008). The authors demonstrate morphological and biochemical changes in brains of mice treated with AlCl₃ combined with D-galactose for 90 days (Rui et al., 2008).

Al, a known neurotoxicant (Miu and Benga, 2006), has been reported to alter the blood-brain barrier (BBB); as a result of which it gains an easy access to the central nervous system (CNS) under normal physiological conditions and accumulates in the different regions of brain (Banks and Kastin, 1989; Exley, 2001; Zatta et al., 2002). It has been reported to be involved in the etiology of several neurodegenerative diseases (Kawahara et al., 2003). Further, Al being a potent cholinotoxin (Gulya et al., 1990) causes apoptotic neuronal loss which is related to high levels of
acetylcholinesterase (AChE) in the brain (Ravi et al., 2000). Latter is associated with
the loss in cognition observed during AD.

Al is reported to potentiate the peroxidative effect of Fe^{2+} (Golub et al., 1999; Oteiza et al., 1993) and this may indirectly indicate its role in causing oxidative damage. Induction of oxidative stress by Al administration is reported to cause damage to
membrane lipids, proteins and antioxidative enzyme defense system (Jyoti and Sharma, 2006; Sethi et al., 2009; Xu et al., 1992). Al also potentiates oxidation caused by other transition metals like chromium (Cr) and copper (Cu) (Bondy et al., 1998). Long term chronic Al-intake is reported to induce oxidative stress related
damages in the CA1 and CA3 field of hippocampus brain regions (Sethi et al., 2008).

It has been proposed to accelerate the aging process by inflicting oxidative damage resulting in biochemical changes (Bharathi et al., 2006). Savory et al. established that aged rabbits are more susceptible to Al toxicity compared to young rabbits (Savory et al., 1999).

In vivo studies report on the apoptosis like changes (Suarez-Fernandez et al., 1999), vacuolated astrocytes with numerous lipofuscin deposits (Florence et al., 1994), abnormal mitochondrial swelling, thinning of myelin sheath, cytoplasm with multivesicular bodies (Deloncle et al., 2001) and synaptic vesicle accumulation (Jyoti and Sharma, 2006) with Al. Numerous epidemiological, neuropathological, and biochemical studies have suggested a possible link between the neurotoxicity of Al and pathogenesis of AD (Kawahara et al., 2001). Further, Al promotes accumulation of insoluble amyloid-β-protein, aggregation of hyperphosphorylated tau-protein which comprises neurofibrillary tangles (NFTs) and also causes detrimental changes to cholinergic neurotransmission (Johnson and Jope, 1986).

There is ample systematic and pragmatic evidence supporting the use of plant-
derived antioxidants for the control of neurodegenerative disorders (Mancuso and Barone, 2009b; Mancuso et al., 2007). Antioxidants may have neuroprotective (preventing apoptosis) and neuroregenerative roles, by reducing or reversing cellular damage and by slowing progression of neuronal cell loss. Although demand for phytotherapeutic agents is growing, and several phytochemicals are reported to show neuroprotection but their delivery across the BBB needs to be confirmed. Latter will be important if we propose to use them in therapeutics. Since most of
these natural molecules show compromised physiological properties and poor pharmacokinetics, we suggest a suitable pharmaceutical couturing of these molecules for effective use in clinical situations (Mancuso and Barone, 2009b).

Curcumin (diferuloylmethane), is a low molecular weight phytochemical, with potent antioxidant and anti-inflammatory activities (Aggarwal and Harikumar, 2009; Frautschy and Hu, 2001). Effectiveness of curcumin has been established in a wide variety of human diseases including neurodegenerative disorders, (Cole et al., 2007; Garcia-Alloza et al., 2007; Ma et al., 2009; Sugiyama et al., 1996) but it is yet to be approved as a therapeutic agent (Banks and Kastin, 1989) due to its poor absorption and stability at physiological pH, rapid metabolism and systemic elimination. Latter factors result in low bioavailability and hence a poor pharmacokinetic profile of curcumin (Calabrese et al., 2008; Mancuso and Barone, 2009a).

Chronic dietary curcumin was reported to lower the amyloid beta (Aβ) deposition in 16-month-old APPsw transgenic mice (Tg2576) (Lim et al., 2001). The progressive accumulation of Aβ aggregates is widely believed to be fundamental to the initial development of neurodegenerative pathology and to trigger a cascade of events such as neurotoxicity, oxidative damage and inflammation that contribute to the progression of AD (Cummings et al., 1998; Pike et al., 1991). Yang et al., reported that like polar Aβ binding compounds such as chrysamine G, curcumin might be able to cross the BBB and bind to amyloid and related aggregates (Yang et al., 2005). However the systemic BA of curcumin is < 1 %; so achieving a significant plasma concentration becomes an important consideration for effective neuroprotective effects of curcumin, including the control of AD like symptoms induced by chronic Al administration. To overcome the compromised physicochemical properties of curcumin, several strategies like use of adjuvants like piperine, nanoparticles, liposomes, complexation with phospholipids and cyclodextrins and solid dispersions have been developed (Anand et al., 2010; Bisht et al., 2007; Maiti et al., 2007; Shoba et al., 1998; Tiyaboonchai et al., 2007; Tsai et al., 2011). Further, to the above quoted strategies, another group reports on synthesis of mitochondria-directed antioxidants, by conjugating curcumin congeners with different polyamine motifs as vehicle tools for achieving improved neuroprotectant effects (Simoni et al., 2010).
SLNs, the lipidic nanoparticles (Kaur et al., 2008) dispersed in a surfactant solution, have been used as suitable drug delivery systems for enhancing the bioavailability of various phytochemical antioxidants like quercitin (Li et al., 2009a) and vinpocetine (Luo et al., 2006). To overcome the poor pharmacokinetics of curcumin and develop it for neurodegenerative disorders, we propose the use of these curcumin loaded solid lipid nanoparticles (C-SLNs).

Present study investigates the therapeutic effect of C-SLNs (50, 25, 12.5 and 1mg/kg) vis-à-vis free curcumin (50 mg/kg) in treatment of biochemical, behavioural and histopathological changes induced after oral administration of AlCl₃ (100 mg/kg).

2.0 MATERIALS AND METHODS

2.1 Animals

Young male Lacca mice (8-12 weeks old; Central Animal House, Panjab University, Chandigarh) weighing 15–25 g were used for the study. Animals were housed under standard (25 ± 2 °C, 60–70 % humidity) laboratory conditions on normal dark and light cycle. Standard rat chow pellets and water was allowed ad libitum. Animals were acclimatized to laboratory conditions before the test. All behavioural experiments were carried out between 10.00 – 17.00 h. The experimental protocols were approved by the Institutional Animal Ethical Committee (IAEC) and conducted according to the guidelines of “Committee for the Purpose of Control and Supervision of Experiments on Animals” (CPCSEA).

2.2 Treatment schedule

The animals were divided into nine groups (n = 10) assigned different treatments (Table 1). All mice (except the naive control group I) were administrated AlCl₃ orally (100 mg/kg) for 12 weeks to set up the AD animal model. Animals were subjected to Morris water maze (MWM) test at 6 and 12 weeks after the start of AlCl₃ treatment, and significant memory lapses were observed at 12 weeks which confirmed the onset of Alzheimer’s like symptoms. Subsequent to 12 weeks of AlCl₃ administration, the mice were treated orally with free curcumin (50 mg/kg) dissolved in 25% tween 80 (FC 50) or with various doses of C-SLNs (SLN 1, 12.5, 25, 50 mg/kg) or blank SLNs (BSLNs) or rivastigmine (1.5 mg/Kg; a standard drug agent used for the control of AD) for 6 weeks. Treatment with AlCl₃ was also continued
during this period. After 6 weeks of respective treatment, mice were subj
MWM test, subsequent to which the animals were sacrificed, and thei
harvested. A 10% brain homogenate was prepared in 150 mM KCl and
biochemical estimations including measurements of acetylcholinesterase
were performed. Histopathological studies were performed for each group, 
brain samples which were preserved in 10% formalin solution.

Table 1. Treatment schedule of various animal groups

<table>
<thead>
<tr>
<th>S.No</th>
<th>Group</th>
<th>Treatment (p.o, mice)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>NAIVE CONTROL</td>
<td>Distilled water for 18 weeks</td>
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<tr>
<td>II*</td>
<td>ALZ</td>
<td>Aluminium chloride (100 mg/kg) for 18 week (12+6) through oral gavage.</td>
</tr>
<tr>
<td>III**</td>
<td>SLN50</td>
<td>C- SLNs at a dose of 50 mg/kg body weight</td>
</tr>
<tr>
<td>IV**</td>
<td>SLN25</td>
<td>C- SLNs at a dose of 25 mg/kg bw</td>
</tr>
<tr>
<td>V**</td>
<td>SLN12.5</td>
<td>C- SLNs at a dose of 12.5 mg/kg bw</td>
</tr>
<tr>
<td>VI**</td>
<td>SLN1</td>
<td>C- SLNs at a dose of 1.0 mg/kg bw</td>
</tr>
<tr>
<td>VII**</td>
<td>FC50</td>
<td>Free curcumin; FC (solution of curcumin in tween 80) at a dose of 50 mg/kg bw</td>
</tr>
<tr>
<td>VIII*</td>
<td>SD (POSITIVE CONTROL)</td>
<td>Standard drug; SD (rivastigmine) at its recommended dose of 1.5 mg/kg bw</td>
</tr>
<tr>
<td>IX**</td>
<td>BSLN</td>
<td>Blank SLNs</td>
</tr>
</tbody>
</table>

*Groups II-IX were administered AlCl₃ for 18 weeks through oral gavage
#Groups III-IX received various treatments for 6 weeks, starting after comp: 12 weeks and upto 18 weeks simultaneously with AlCl₃ treatment

2.3 Behavioural assessments

2.3.1 Assessment of cognitive performance

2.3.1.1 Morris water maze test (MWM)

Animals were tested in a spatial version of MWM (Morris et al., 1982; Tu Baydas, 2006). The maze consisted of a circular pool (1.2 m in diameter and high) made of iron and coated with a black non-toxic paint (Van der Staa}
Jonge, 1993). The pool was filled to a depth of 20 cm with water (24-25°C). An escape platform (10 cm in diameter), made of iron painted black with a grooved surface for a better grip, was submerged 0.5 cm under the water level. The animal has to swim until it finds the hidden platform. The tank was located in a large room where there were coloured cues external to the maze and could be used by the mice for spatial orientation. The position of the cues remained unchanged throughout the study. The water maze task was carried out for five consecutive days starting from day one. The mice received four consecutive daily training trials with each trial having a ceiling time of 90 s and a trial interval of approximately 30 s. For each trial, mice were put into the water at one of the four starting positions, in a random sequence. During test trials, mice were placed into the tank at the same starting point, with their heads facing the wall. The mice had to swim until it reached onto the platform submerged in the water. After climbing onto the platform, the animal remained there for 20 s before the commencement of the next trial. The escape platform was kept in the same position relative to the distal cues. If the mice failed to reach the escape platform within the maximally allowed time of 90 s, it was gently placed on the platform and allowed to remain there for the same amount of time. On the fifth day the time to reach the platform (escape latency in seconds) was measured. During the observational training, a single observer watched the animals in MWM tank without interacting with the other observers. The acoustic and mechanical stimuli delivered to the animals were successful in maintaining a good level of alertness.

2.4 Biochemical assessment

Biochemical tests were conducted 24 h after the last behavioural test. The animals were killed by decapitation. Brains were removed and rinsed with ice-cold isotonic saline and then homogenised with ice-cold 150 mM KCl. The homogenate (10% w/v) was centrifuged at 10,000 rpm for 15 min. and the supernatant so formed was used for biochemical estimations.

2.4.1 Acetylcholinesterase (AChe) activity

Cholinergic dysfunction was assessed in terms of acetylcholinesterase activity. The quantitative measurement of acetylcholinesterase levels in brain were performed according to the method of Ellman (Ellman and Courtney, 1961). The assay mixture contained 0.05 ml of supernatant, 3 ml of 0.01 M sodium phosphate buffer (pH 8),
0.10 ml of acetylthiocholine iodide and 0.10 ml 5,5', dithiobis-(2-nitro benzoic acid) (Ellman reagent). The change in absorbance was measured at 412 nm for 5 min. Results were calculated using molar extinction coefficient of chromophore (1.36 × 10^4 M⁻¹ cm⁻¹) and expressed as percentage of control.

### 2.4.2 Measurement of lipid peroxidation

The extent of lipid peroxidation in the brain was determined quantitatively by the method by Wills (Wills, 1966). The amount of thiobarbituric acid reactive substances (TBARS) was measured by reaction with thiobarbituric acid at 532 nm using Perkin Elmer Lambda 20 spectrophotometer. The values were calculated using the molar extinction coefficient of chromophore (1.56 × 10^5 M⁻¹ cm⁻¹).

### 2.4.3 Estimation of reduced glutathione

Reduced glutathione was assayed by the method of Jollow et al. (Jollow et al., 1974). Briefly, 1.0 ml of supernatant (10%) was precipitated with 1.0 ml of sulphosalicylic acid (4%). The samples were kept at 4°C for at least 1 h and then subjected to centrifugation at 1,200 g for 15 min at 4°C. The assay mixture contained 0.1 ml supernatant, 2.7 ml phosphate buffer (0.1 M, pH 7.4) and 0.2 ml 5’ dithiobis-(2-nitro benzoic acid) (Ellman’s reagent, 0.1 mM, pH 8.0) in a total volume of 3.0 ml. The mixture developed a yellow color which was read immediately at 412 nm.

### 2.4.4 Estimation of superoxide dismutase

Superoxide dismutase activity was assayed by the method of Kono (Kono, 1978). The assay system consisted of 0.1 mM EDTA, 50 mM sodium carbonate and 96 mM of nitroblue tetrazolium (NBT). In the cuvette, 2 ml of above mixture was taken and to it 0.05 ml of supernatant and 0.05 ml of hydroxylamine hydrochloride (adjusted to pH 6.0 with NaOH) were added. The auto-oxidation of hydroxylamine was observed by measuring the change in optical density at 560 nm for 2 min at 30/60 s intervals.

### 2.4.5 Estimation of catalase

Catalase activity was assayed by the method of Claiborne (Claiborne, 1985). Briefly, the assay mixture consisted of 1.95 ml phosphate buffer (0.05 M, pH 7.0), 1.0 ml hydrogen peroxide (0.019 M) and 0.05 ml supernatant (10%) in a final volume of 3.0 ml. Changes in absorbance were recorded at 240 nm. Catalase activity was calculated in terms of amount of H₂O₂ decomposed per min and expressed as mean ± S.E.M.
2.4.6 Blood lipid profile

After 6 weeks of treatment post induction of Alzheimer’s like symptoms, the blood of the animals of control group, AlCl₃ administered group, free curcumin and C-SLNs (50 mg/kg) treated groups were withdrawn through sinus under clavicle into heparinised vials and observed for the lipid profile. Latter included measurement of serum cholesterol (%mg), high density lipoprotein (HDL) cholesterol (%mg), low density lipoprotein (LDL) cholesterol (Direct; %mg), very low density lipoprotein (VLDL) cholesterol (%mg), CHOL/HDL ratio, LDL/HDL ratio, triglycerides (%mg), and phospholipids (%mg).

2.5 Histopathological analysis

Animals were sacrificed and brains were harvested. For morphological observation by light microscopy, brain tissue was fixed in 10% neutral formaldehyde solution, and embedded in paraffin. Paraffin sections of thickness 7 mm were prepared for microscopic study. Finally tissues were dipped in water and then transferred to crystal violet stain for 10 min at 60°C.

The stained sections were washed in running water to remove excess stain and then upgraded for dehydration through different grades of alcohol. Slides were cleared with xylene and mounted with DPX to make them permanent. The slides were observed under Nikon 80 i at 400X. A minimum of 3-4 sections were prepared for each brain sample. The sections were observed for the presence of vacuolization/spongiosis, lipofuscin deposits and neuronal degeneration.

3.0 STATISTICAL ANALYSIS

All data was analysed via one-way analysis of variance (ANOVA) using SigmaStat 2.0 software (Jandel Scientific); presented data are mean ± SEM (n=3). A value of p ≤ 0.05 was considered significant.

4.0 RESULTS

4.1 Behavioural assessment

4.1.1 Morris water maze test for spatial memory

Aluminium chloride-treated mice showed an initial increase in escape latency, which continued during the training for spatial navigation task. There was a significant
difference in the mean escape latency of the Al treated group when compared to the control group on day 90 (12 weeks) indicating that chronic administration impaired acquisition of spatial navigation task ($P \leq 0.05$). Similar results are reported by other researchers (Platt et al., 1995; Platt et al., 2001). Further, C-SLNs (25 and 50 mg/kg) and free curcumin treatment coupled with continued administration for another 6 weeks showed a significant decrease in escape latency to reach the platform (Figure 1). It may be noted that the improvement in the cognitive abilities achieved with free curcumin (50 mg/kg) was significantly less compared to those achieved with all the doses of C-SLNs. Furthermore, the attenuation of cognitive impairments was observed to be complete (i.e., the values were similar to the naive control) even at the lowest dose of 1 mg/kg. The results obtained with C-SLNs at all doses were comparable ($p<0.001$) to those of standard anti-AI drug, rivastigmine (SD Group).

![Figure 1. Effect of C-SLNs (1, 12.5, 25, 50 mg/kg) and free curcumin treatment on memory performance in Morris water maze test](image)

Each data point represents the mean (S.E.M.) escape latency of 6 trials for each mice.

*All the values are significantly ($p<0.05$) different than the control.

# Values restored to naive control.
4.2 Biochemical assessment

4.2.1 Acetylcholinesterase (AChE) activity

AChE activity was observed to be elevated in Al-intoxicated mice. However, treatment with C-SLNs could completely mitigate the AChE levels to normal (p≤0.1), while free curcumin could achieve a recovery of not more than 22.23%. Protective effects of curcumin in Al induced elevation of AChE levels have been reported earlier (Zatta et al., 2002; Zatta et al., 1994). Results as shown in Table 2, demonstrate that the effects obtained with 1 mg/kg dose of C-SLNs are similar to those obtained with rivastigmine (p≤0.1). Results obtained with free curcumin though not significantly different have arithmetically low values as compared to rivastigmine and C-SLNs (at all doses). A very interesting observation is a significant effect obtained with blank SLNs. Later may be correlated well to the similar observations obtained in glutathione levels as explained subsequently in the biochemical assessment section.

4.2.2 Lipid peroxidation, reduced glutathione, SOD and catalase

Therapeutic effects of C-SLNs on aluminium-induced biochemical modifications in mice brain homogenates were evaluated. In Al treated mice, the levels of TBARS were significantly (p≤0.05) elevated but the activities of glutathione, superoxide dismutase, and catalase were significantly lower in the brain homogenates (Table 2). These observations are similar to earlier reports (Kawahara et al., 2003) indicating oxidative stress in Al-treated groups attributable to the peroxidation of the brain lipids (Newairy et al., 2009; Yousef et al., 2005). The increased lipid peroxidation is, at least in part, due to an inhibition of superoxide dismutase (Kawahara et al., 2003) and catalase (Fridovich, 1975) activities in the brain as observed by us in Table 2. A similar reduction in reduced GSH enzyme activity was observed after AlCl₃ treatment. However, subsequent treatment with C-SLNs and blank SLNs restored the GSH levels close to the naïve control values (p≤0.001). Significant effects obtained with blank SLNs group is reported and discussed by us very recently in the management of anxiety and memory loss associated with menopause in an ovariectomised model of rat (Kakkar et al., 2011a).
Table 2. Effect of free curcumin (FC) and C-SLNs on aluminium chloride induced oxidative stress parameters in mice brain

<table>
<thead>
<tr>
<th>Groups</th>
<th>AChE (umoles of acetylthiocholine hydrolysed/mg protein)</th>
<th>LPO (nmole MDA/mg protein)</th>
<th>Catalase (nmole of H₂O₂ decomposed/mg protein)</th>
<th>SOD (Units/protein)</th>
<th>GSH (amount utilized/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NAIVE CONTROL</td>
<td>0.11±0.01</td>
<td>1.18±0.17</td>
<td>6.47±1.11</td>
<td>0.47±0.04</td>
<td>0.33±0.6</td>
</tr>
<tr>
<td>ALZ</td>
<td>0.22±0.03*</td>
<td>3.00±0.35*</td>
<td>2.17±0.18*</td>
<td>0.05±0.05*</td>
<td>0.09±0.0</td>
</tr>
<tr>
<td>SLN50</td>
<td>0.14±0.01*</td>
<td>1.15±0.24*</td>
<td>7.88±0.03*</td>
<td>0.31±0.06*</td>
<td>0.26±0.0</td>
</tr>
<tr>
<td>SLN25</td>
<td>0.12±0.01*</td>
<td>1.17±0.19*</td>
<td>6.12±1.10*</td>
<td>0.33±0.06*</td>
<td>0.22±0.0</td>
</tr>
<tr>
<td>SLN12.5</td>
<td>0.10±0.02*</td>
<td>1.55±0.23*</td>
<td>6.86±0.88*</td>
<td>0.28±0.06</td>
<td>0.23±0.0</td>
</tr>
<tr>
<td>SLN1</td>
<td>0.17±0.01*</td>
<td>1.62±0.10*</td>
<td>5.01±0.38*</td>
<td>0.29±0.03*</td>
<td>0.25±0.0</td>
</tr>
<tr>
<td>FC50</td>
<td>0.18±0.04</td>
<td>2.56±0.12</td>
<td>3.67±0.62</td>
<td>0.13±0.01</td>
<td>0.17±0.0</td>
</tr>
<tr>
<td>SD</td>
<td>0.17±0.02</td>
<td>0.91±0.02</td>
<td>7.46±0.12</td>
<td>0.38±0.01</td>
<td>0.26±0.0</td>
</tr>
<tr>
<td>BSLN</td>
<td>0.12±0.03*</td>
<td>2.66±0.31*</td>
<td>2.02±0.14</td>
<td>0.07±0.01</td>
<td>0.22±0.0</td>
</tr>
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</table>

*All the values are significantly different (p<0.05–p<0.1) from Naive Control
# Values were restored to Naive control post treatment with C-SLNs (p<0.05)

4.2.3 Blood lipid profile

Our study showed that treatment of mice with aluminium chloride decreased triglycerides and VLDL in the mice blood (Table 3). C-SLNs treatment could help normalise the lipid profile of the blood to the normal values.

Table 3. Change in blood lipid profile of mice, after treatment with curcumin vis-à-vis C-SLNs

| Groups         | Serum Cholesterol (mg%) | HDL Cholesterol (mg%) | LDL Cholesterol (Direct) (mg%) | VLDL Cholesterol (mg%) | CHOL/HDL Ratio (mg%) | LDL/HDL Ratio (mg%) | Triglycerides (mg%) | Prec. (
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<tbody>
<tr>
<td>Normal</td>
<td>96</td>
<td>48</td>
<td>23.80</td>
<td>24.2</td>
<td>2</td>
<td>0.5</td>
<td>121</td>
<td></td>
</tr>
<tr>
<td>Alz Model</td>
<td>91</td>
<td>59</td>
<td>25.80</td>
<td>6.20</td>
<td>1.54</td>
<td>0.4</td>
<td>31</td>
<td>1</td>
</tr>
<tr>
<td>Free Curcumin</td>
<td>80</td>
<td>46</td>
<td>8.60</td>
<td>25.40</td>
<td>1.74</td>
<td>0.2</td>
<td>127</td>
<td>1</td>
</tr>
<tr>
<td>(50 g/kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-SLNs</td>
<td>88</td>
<td>46</td>
<td>16.40</td>
<td>25.60</td>
<td>1.91</td>
<td>0.4</td>
<td>128</td>
<td>1</td>
</tr>
<tr>
<td>(50 mg/kg)</td>
<td></td>
<td></td>
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</table>
4.3 Histopathological analysis

Histological observations indicate that AlCl₃ treated groups exhibited disrupter neurons in the area of edema, degenerated neurons, vacuolization around the neuron/perineuronal space/spongiosis, disruption of nucleus and congestion in the blood vessels (Figure 2B). Similar histochemical changes have also been reported by others researchers in the field.

Figure 2. Microscopic study of lateral sections of mice brain (400x). Histological sections of brain were stained with hematoxylin and eosin (H and E).
5.0 DISCUSSION

The present study was carried out to investigate the protective effect of developed C-SLNs in comparison to free curcumin in alleviating AlCl₃ induced oxidative stress and biochemical alterations in mice brain. Further, changes in whole brain histopathology and blood lipid profile were also examined.

Aluminium is a ubiquitous metal that has been implicated in the aetiology of neurodegenerative disorders where it exacerbates brain oxidative damage (Nehru et al., 2007). It is characterized by impairment in working memory (Germano and Kinsella, 2005), visuoperception, attention and semantic memory. During the induction of AD using aluminium chloride in mice, we very astonishingly ended with a small number of animals (3-4) per group, due to a high mortality rate (>40 %). Surprisingly, this fact has never been reported in the literature, although discussion with several other workers in the area indicated a similar practical experience.

Oral administration of aluminium chloride has been shown to cause learning deficits in Morris water maze task in rats (Walton, 2007b). Chronic aluminium treatment is also known to cause impairment of glutamate–NO–cGMP pathway in the cerebellum of rats (Canales et al., 2001), resulting in memory losses and neurobehavioural deficits. Cognitive deficit associated with aluminium administration can also be attributed to the glial reaction and pathological changes reported in the cholinergic fibres of rat brain (Platt et al., 2001).

MWM test results clearly demonstrated declined spatial learning abilities in aluminium-treated rats. A marked decrease in escape latency time during ongoing acquisition trial indicates retrieval of memory in MWM test post treatment with C-SLNs. Further, even low dose of 1 mg/kg could result in reversal of the memory losses incurred as a result of chronic administration of aluminium chloride.
Part 1: Chapter V

Acetylcholine (Ach), a neurotransmitter associated with learning and memory, is degraded by the enzyme acetylcholinesterase, terminating the physiological action of Ach. In addition to their role in cholinergic transmission, cholinesterases may also play a role during morphogenesis and neurodegenerative diseases (Reyes et al., 1997). It is proposed by Zatta et al. (Zatta et al., 1994) that the observed elevated activity of AChE could be due to a direct effect of Al(III). They claim that Al(III) can interact with the peripheral sites of AChE and modify its secondary structure and eventually its activity. Following experimental AlCl₃ exposure, significant increase in lipid peroxidation of brain tissue of rat pups were observed (Nehru and Anand, 2005). Al stabilizes iron in its reduced ferrous (Fe²⁺) state (Sakamoto et al., 2004; Yoshino et al., 1999) and promotes Fe²⁺-initiated lipid peroxidation by the Fenton reaction in a dose-dependent and time-dependent manner (Verstraeten et al., 1994).

Al, a non-redox-active metal, shows pro-oxidant activity both in vitro and in vivo (Exley, 2004). The increased lipid peroxidation observed as a result of Al administration may be attributed to an inhibition of superoxide dismutase (Kawahara et al., 2003) and catalase (Fridovich, 1975) activities in the brain as observed by us in Table 1. This deprivation of the endogenous antioxidant system results in a substantial increase in the rate of phospholipid peroxidation in brain cells, leading to membrane damage and neuron death. SOD presents the first line of defence against the produced superoxides, as it dismutases the superoxide anion to H₂O₂ and O₂ (Fridovich, 1975). Because the SOD enzyme generates H₂O₂, it works in collaboration with H₂O₂ removing enzymes e.g catalase which converts H₂O₂ to water and oxygen. Catalase is present in the peroxisomes of mammalian cells, and probably serves to destroy H₂O₂ generated by oxidase enzymes located within these sub cellular organelles (Walton, 2007). A similar reduction in reduced GSH enzyme activity was observed after AlCl₃ treatment. Depletion in GSH levels with Al are also reported in an earlier study (Sethi et al., 2008b). Restoration of GSH levels by C-SLN and blank SLNs close to the naïve control values (p≤0.001) may be assigned to the intake of phosphatidylcholine.

Since, our formulation contained soy lecithin, which consists of 21% phosphatidylcholine and was administered continuously for a time period of 6 weeks, it could probably lead to an increase in the levels of GSH. GSH levels in the brain have a direct influence on memory (linked to acetylcholinesterase) (Shukitt-Hale et
al., 1998). Hence a significant attenuation of AChE as observed with blank SLNs could also be due to an improvement in GSH levels, and indirectly due to phosphatidylcholine present in blank SLNs.

Treatment with C-SLNs significantly attenuated the oxidative stress induced biochemical alterations in Al–treated rat brain homogenates to the naïve control values and the effect was significantly better than that observed with free curcumin which could attenuate the oxidative stress by not more than 40% with respect to Al treated group. A small effect achieved with free curcumin could be assigned to the disruption of BBB due to Al toxicity such that free curcumin also gains an easy access to the brain. However limited absorption of free curcumin across the gut and its proneness to metabolic transformation in the gut and liver may be the reason for a lower effect. An effect obtained with even low dose (1 mg/kg) of C-SLNs indicates that even minute quantities of curcumin can elicit an effect i.e the rate limiting step is permeability across and delivery to the brain and not the concentration achieved in the brain. It seems that curcumin triggers the cascade of events which result in neuroprotection once it reaches the brain. This is expected out of neurohormetics and curcumin is proposed as a neurohormetic in in vitro experiments (Calabrese and Baldwin, 2002; Kakkar and Kaur, 2011a; Kakkar and Kaur, 2011b).

Administration of C-SLNs, according to us helps improve not only the permeability of curcumin across the BBB (which otherwise is also disrupted due to AlCl₃ administration) but also helps in membrane stabilisation. It is purported that this positive interaction of nanoparticles with neuronal and astrocyte membranes would not only provide integrity to the membrane, but would also help restore the disrupted BBB. Latter may now restrict the entry of Al as well as other macromolecules which are expected to be toxic, but the passage of the solid lipidic nanoparticles of curcumin continues thus highlighting the importance of formulating curcumin.

Decreased triglycerides and VLDL in the mice blood post treatment of mice with aluminium chloride was observed. A similar observation regarding reduced TG upon Al administration is reported in rats (Sugawara et al., 1988 ) and in humans (Lepara et al., 2009). It has been shown that cholesterol modulation may affect generation of Aβ and hence can be a possible cause of AD (Koudinov and Koudinova, 2000). Further to this it is reported that this impairment could be reversed by restriction of cholesterol rich diet (and its replacement with a regular diet) for an extended period of time. Baum et al., indicated that any decrease in cholesterol levels, attained with
curcumin may be due to inhibition of dietary cholesterol absorption and not because of its effects on cholesterol synthesis (Baum et al., 2007). Thus, it may be concluded that curcumin is exerting its major effect in the gut and hence no difference in improved lipid profiles is observed for free curcumin and C-SLNs groups. It may however be concluded from these reports, that Al administration alters the lipid profile in the physiological system, which is restored upon curcumin administration.

A disruption of neuronal and perineuronal areas observed in the Al treated mice, upon histopathological examination is consistent with other reports. A study demonstrated a dense cytosolic staining due to increased lipofuscin accumulation in Al toxicity (Jyoti and Sharma, 2006). In another study report on Al intoxicated mice authors reported similar findings in the brains of experimental animals, studied by optical microscopy. They confirmed a damage in the hippocampus and cortex, including neurofibrillary degeneration, due to the accumulation of Al in these regions (Rebai and Djebli, 2008).

Histopathology of brain sections of SLN treated mice illustrated near normalisation of the brain microstructural elements, with normal neurons and intact nucleus and astrocytes. No spongiosis/vacoulsation and degenerated neurons could be observed (Figure D-F). These effects were evident even at the lowest dose of C-SLNs while free curcumin group did not show significant improvement.

These findings provide further compelling evidence that aluminium promotes alterations in behavioural, biochemical and histopathological changes, via oxidative mechanism and C-SLNs with their enhanced BA may be a useful therapeutic agent to treat Alzheimer’s disease.

6.0 CONCLUSION

Our results report alterations in brain histopathology, loss of cognition and an oxidative damage induced biochemical changes, including inhibition of antioxidant enzyme activities as a result of AlCl₃ exposure. Curcumin administered in its highly bioavailable form (C-SLNs) completely reversed the induced alterations. This gives a direct evidence of effective brain delivery of C-SLNs and for the first time establishes the curative-therapeutic role (rather than a mere protective role established by simultaneous or prior administration) of curcumin, in AD like symptoms and induced cognition losses, upon its incorporation into SLNs.