Chapter-6

Cineole prevented Aβ induced toxicity in PC12 cells
**Introduction**

Alzheimer’s disease (AD) is a progressive brain disorder affecting regions that control memory and cognitive functions, gradually destroying a person’s memory and ability to learn, to communicate, to reason, and to carry out daily activities. The two major neuropathological hallmarks of AD are extracellular amyloid beta (Aβ) plaques and intracellular neurofibrillary tangles. It is estimated that 27 million people are affected worldwide by AD (Wimo et al., 2006). As population life expectancy increases, the number of affected individuals is expected to triple by 2050 (Hebert et al., 2003). The major cause and the pathways involved in AD pathogenesis are not fully understood but oxidative stress and neuroinflammation are being suggested as the major ones. The link between Aβ and neurodegeneration has not yet explored but to some extent neuroinflammation could provide this missing link. It has been found that gliosis is the common phenomenon in AD pathogenesis. Clusters of activated microglia and macrophages have been detected in senile plaques (Rogers et al., 1988). The full amatory of the humoral inflammation, e.g., cytokines, chemokines, complement and coagulation factors or adhesion molecules can be found in AD lesions (Griffin et al., 1995).

Inflammation is a response to eliminate the initial cause of cell injury as well as the necrotic cells and tissues resulting from the original insult. It is unclear, whether inflammation in AD is detrimental, beneficial, or an irrelevant epiphenomenon. Brain inflammation is the pathological hallmark of AD. The characteristic inflammatory features such as swelling, heat, and pain are not seen in the brain, and therefore we refer it chronic inflammation (Akiyama et al., 2000a). Mononuclear phagocytes (that include microglia) and astrocytes are crucial in host defense to infection and injury and have the potential to injure and even kill contiguous neurons by release of highly toxic products, such as reactive oxygen species (ROS), nitric oxide (NO), inflammatory cytokines, proteolytic enzymes, complement factors, or excitatory amino acids. Therefore, chronic release of neurotoxic factors triggered, e.g., by Aβ deposits could explain the progressive neurodegeneration in AD. 1,8 cineole (Cin), is an essential oil present in large amounts in a variety of
plants. It is often employed by the pharmaceutical industry in drug formulations, as a percutaneous penetration enhancer, as a nasal decongestant and anticough agent, in aromatherapy, and in dentistry (Dogan et al., 2001; Uemura et al., 1997; Laude et al., 1994; Weyers and Brodbeck, 1989). Cin has been used to treat bronchitis, sinusitis and chronic rhinitis and also for the treatment of asthma (Juergens et al., 1998a). Cin also inhibits the production of cytokines and prostaglandins by stimulated monocytes in vitro, explaining its bronchodilator effect (Juergens et al., 1998b). More recently Santos and Rao, (2000) reported the antiinflammatory and antinociceptive effects of Cin.

In the light of these observations, it has created our interest to evaluate the possible antiinflammatory effect of cineole in differentiated PC12 cells toxicated by amyloid beta_{25-35} (Aβ_{25-35}).

**Materials and Methods**

**Chemicals and reagents**

As described in materials and methods, chapter III

**Cell culture**

As described in materials and methods, chapter III

**Treatment**

Cells were cultured in flasks or plates. After 8 days of differentiation, the medium was replaced and fresh medium containing Cin was added for 24 h. Thereafter, the fresh medium containing Aβ_{25-35} was added for 24 h replacing medium containing Cin.

**Cytotoxicity and cell viability**

As described in materials and methods, chapter III

**Biochemical estimation**

The biochemical assays (LDH, ROS, MMP, NO, cytokines and protein) are described in materials and methods chapter III.

**Immunocytochemistry**

As described in materials and methods chapter III.

**Statistical analysis**

As described in materials and methods chapter III.

**Results**
Determination of PC12 cells viability with different concentrations of Aβ_{25-35} and Cin

The cytotoxicity of Aβ_{25-35} was evaluated which is based on its effect on cell growth (MTT Assay). Fig. 6.1 deals with PC12 cells exposed with Aβ_{25-35} at a series of concentrations (0, 0.01, 0.1, 1, 10, 20 and 40 µM) for 24 h. In Fig. 6.1, Aβ_{25-35} induced marked decrease in cells viability in a dose dependent manner and 10 µM Aβ_{25-35} significantly reduced cells viability to 54.12 ± 3.81% (p < 0.05). The possible cytotoxicity of cin was evaluated which also based on the cells growth by MTT and NRU assays (Fig.6.2). Differentiated PC12 cells were exposed to varying concentrations of Cin (0-50 µM) for 24 h. Fig 6.2 shows that IC_{50} for Cin by MTT was 20 µM while IC_{50} by NRU was 18.8 µM. Thus for further experiments higher cytotoxic doses were not used.

Cineole pretreatment decreased the activity of lactate dehydrogenase in Aβ_{25-35} treated PC12 cells

LDH release measure the membrane integrity as a function of the amount of cytoplasmic LDH released into the medium. This is a marker of cell damage. Aβ_{25-35} (10 µM) significantly increased the activity of LDH (p < 0.01) which was restored by 5 µM and 10 µM Cin (p < 0.05) (Fig. 6.3). Therefore, three non toxic doses of Cin
were selected for further studies viz. 2.5 µM, 5 µM and 10 µM.

**Protective effect of Cin on mitochondrial membrane potential (ψm) in Aβ25-35 treated PC12 cells**
Mitochondrial membrane potential in stressed cells was disrupted which was used to study the mitochondrial health. JC-1 dye was used as the marker of membrane disruption. The green/red ratio was found to be increased significantly in Aβ25-35 treated cells (p < 0.001) as compared to control group suggesting decreased membrane potential. Cineole pretreatment augmented the membrane potential significantly (p < 0.01) in both higher doses as compared to Aβ25-35 treated cells (Fig. 6.4).

**Cineole protects the intracellular oxidative stress in Aβ25-35 treated PC12 cells**
Apart from the anti inflammatory effect of Cin, it also possesses antioxidative effect (Ciftci et al., 2011). To clarify this, the accumulation of ROS was evaluated.

As illustrated in Fig. 6.5, there was a significant increase in the intracellular ROS (p < 0.001) in cells treated with Aβ25-35 compared to control taken as 100%. Cin (5 µM) + Aβ (p < 0.05) and Cin (10 µM) + Aβ pretreatment has decreased the ROS level significantly (p < 0.01) when compared to Aβ25-35 treated cells.

**Attenuation of NO level by Cin in Aβ25-35 treated PC12 cells**
Fig. 6.6 shows significant increase (p < 0.01) of NO level in Aβ25-35 treated cells as compared to control group. Cin
(2.5 μM) + Aβ (p < 0.05), Cin (5 μM) + Aβ (p < 0.05) and Cin (10 μM) + Aβ significantly protected (p < 0.01) the toxicity induced by Aβ25-35.

Recent findings suggested the role of inflammation in AD (Hochstrasser, 2013; Koyama 2012). The Aβ25-35 treated cells caused a significant elevation in proinflammatory cytokines, TNF-α (p < 0.001), IL-1β (p < 0.001) and IL-6 (p < 0.001) as compared to the control. Cin pretreatment significantly attenuated the level of TNF-α at higher doses (5 μM and 10 μM) (p < 0.05 and p < 0.01 respectively) as compared to Aβ25-35 treated cells (Fig. 6.7). Cin (5 μM) + Aβ25-35 showed depletion in IL-1β level (p < 0.05) while Cin (10 μM) + Aβ25-35 showed more pronounced attenuation (p < 0.01) as compared to Aβ25-35 treated PC12 cells (Fig. 6.8). IL-6 level was also attenuated significantly by
Cin (2.5 μM) + Aβ_{25-35} (p < 0.05), Cin (5 μM) + Aβ_{25-35} (p < 0.05) and by Cin (10 μM) + Aβ_{25-35} (p < 0.01) as compared to Aβ_{25-35} treated cells (Fig 6.9).

Cineole pretreatment attenuated the expressions of COX-2, NOS-2, NF-κB and ChAT

The up regulation of COX-2, NOS-2 and NF-κB is linked with AD pathogenesis. The expression of COX-2, NOS-2 and NF-κB was increased in PC12 cells treated with Aβ_{25-35} (Fig. 6.10. B, E, H). In Cin (10 μM) + Aβ_{25-35} treated cells, the expression was decreased as compared to Aβ_{25-35} treated cells (Fig. 6.10. C, F, I). Only Cin (10 μM) did not show any marked effect on the cells as compared to control group (data not shown).

ChAT expression was decreased in Aβ_{25-35} treated PC12 cells (Fig. 6.10. K) as compared to control which was attenuated significantly in Cin (10 μM) + Aβ_{25-35} treated cells as compared to Aβ_{25-35} treated cells (Fig. 6.10 L).

Discussion

AD is a neurological disorder that presently affects 20-30 million individuals around the world (Selkoe, 2005). Generation and deposition of Aβ peptides and neurofibrillary tangle formation are key mechanisms involved in AD pathogenesis. Recent evidence suggests that inflammatory mechanisms represent a third component which, once initiated by degeneration, may significantly contribute to disease progression and chronicity (Heneka and O'Banion, 2007). Neuroinflammation is now recognized as a prominent feature in Alzheimer’s pathology and a potential target for therapy aimed at treatment and prevention of disease (Akiyama, 2000a).

CNS was traditionally thought to be immunologically privileged organ but today it is known to have an endogenous immune system that is coordinated by immunocompetent cells such as the microglia (Tuppo and Arias, 2005). The major players involved in the inflammatory process in AD are thought to be the microglia and the astrocytes and possibly to a less extent the neurons, all of which are cellular components of the brain (Heneka and O'Banion, 2007; Akiyama et al., 2000a; Akiyama et al., 2000b).

In the present study the effect of Aβ_{25-35} on MMP and LDH was determined. It was observed that there was
Figure 6.10. Effect of Cin pretreatment on COX-2, NOS-2, NF-κB and ChAT expression. The profound expression of COX-2, NOS-2 and NF-κB was observed in Aβ 25-35 group while expression of ChAT was decreased in Aβ 25-35 groups (B, E, H, K) compared to control groups (A, D, G, J), while Aβ 25-35 group pretreated with Cin (C, F, I, L) has shown a moderate expression of COX-2, NOS-2, NF-κB and ChAT.

significant increase in MMP and release of LDH in Aβ 25-35 treated cells in accordance with the previous reports (Khan et al., 2012). Membrane integrity was lost due to the treatment with Aβ 25-35 which was significantly restored by Cin pretreatment. These results suggest that Cin act as a protective agent for the cellular integrity.

Further, test of cognitive function is very important in AD pathology (Khan et al., 2012) which is represented by cholinergic pathway in the brain. Hence, we tested the expression of choline acetyltransferase (ChAT) and observed its downregulation in cells treated with Aβ 25-35 which was restored to normal by Cin pretreatment. Moreover, the number of cells also
decreased in Aβ25-35 treated group as compared to control which was significantly increased in Cin treated cells.

Aβ induces the expression of proinflammatory cytokines along with increase in ROS and NO in glial cells in a vicious cycle (Lindberg, 2005; Griffin, 1998) and the induction of inflammatory enzyme systems such as the inducible nitric oxide synthase (NOS-2) and the cyclooxygenase enzyme (COX-2). This has been supported by the results of the present study where Aβ25-35 treatment leads to release of ROS, NO and cytokines and upregulation of NOS-2, COX-2 in differentiated PC12 cells. Cin pretreatment has shown prevention in the generation of ROS and NO which determines it as a potent antioxidant and anti inflammatory agent. Further NOS-2 and COX-2 expressions were also restored to normal by Cin pretreatment in PC12 cells.

The mechanism by which Aβ stimulates glial cells is still unclear; however, there is evidence that the peptide induces activation of nuclear-factor κB (NF-κB) (Bales, 2000; Akama, 1998), a transcription factor implicated in the induction of numerous genes, including cytokines. Activation of this pathway depends upon the phosphorylation dependent removal of inhibitory subunit called IκB from the transcription factor NF-κB. This hypothesis is confirmed by the results of the present study where the treatment of cells with Aβ25-35 leads to upregulation of NF-κB which was restored significantly by Cin pretreatment. Hence this could be suggested that Cin has prevented the upregulation of NF-κB suggesting its beneficial role in neuroinflammation.

Upregulation of NF-κB is known to secrete different cytokines and in turn gets activated by them (Kim et al., 1999). This vicious cycle keeps on working which increased the load on the cells. To confirm this we have also analyzed the cytokines profile. Nearly all the cytokines and chemokines that have been studied in AD, including IL-1β, IL-6 and TNF-α seem to be upregulated in AD compared with control individuals (Akiyama et al., 2000a). The production of interleukins and other cytokines and chemokines may also lead to microglial activation, astrogliosis, and further secretion of proinflammatory molecules and
amyloid, thus perpetuating the cascade (Ho et al., 2005). Microglial-derived IL-1β appears to be a driving force in the process of activation of procytokines. IL-1β has been previously shown to be a potent immunomodulating cytokine that induces multiple inflammatory mediators in astrocytes and neurons (Mrak, 1995). It’s overexpression is a consistent feature of post-mortem AD brain (Griffin 1995; Mrak, 1995, Griffin, 1989). TNFα, another pro-inflammatory cytokine secreted primarily by activated macrophages and microglia, is known to promote cell survival and death in the CNS (Stoll et al., 2000).

Immunohistochemical studies show an increase in microglial TNFα localized to senile plaques, suggesting its participation in Aβ-induced inflammation (Dickson et al., 1993). Recent research has demonstrated that TNFα is essential for Aβ-induced neurotoxicity (Viel et al., 2001; Combs et al., 2000). IL-6 is consistently detected in the brains of Alzheimer's disease patients but not in the brains of non-demented elderly persons. Appearance of IL-6 may precede neuritic changes and is not just a consequence of neuritic degeneration. This suggests that activation of inflammatory mechanisms may cause neuritic degeneration in plaques (Hull et al., 1996). These studies suggested the increased expression of cytokines in AD brain. In the present study, there was marked increase in the expression of cytokines (TNF-α, IL-1β, and IL-6) treated with Aβ25-35. The pretreatment with Cin has prevented this increase in accordance with the previous studies (Serafino et al., 2008, Juergens et al., 2004).

**Conclusion**

The present study summarizes the anti-inflammatory role of Cin to ameliorate the adverse condition of the cells treated with Aβ25-35. Our results indicated that Cin has potential to mitigate the toxic effects induced by Aβ25-35 on differentiated PC12 cells. Although many inflammatory mediators are incorporated in this study, further extensive experiments in this direction are needed to proceed for clinical therapy of AD by Cin.
Figure 6.11. Summarized diagram of the prevention of Cin on amyloid beta induced toxicity in PC12 cells: This figure shows the induction of oxidative stress and NO production with increase in NOS-2, COX-2 and NF-κB expression by Aβ25-35 toxicity. Briefly, PC12 cells exposed to Aβ25-35 results in oxidative stress as evident from increase in ROS. Aβ25-35 insult also leads to mitochondrial damage and results in decrease mitochondrial membrane potential ($\psi_m$) along with the increase in cytokines production. In the present study, we demonstrated that Cin effectively decreased the oxidative stress and NO level and increased cytokines and $\psi_m$. Cin has also downregulated the expressions of NOS-2, COX-2 and NF-κB thereby suggesting its preventive role.