Introduction

Alzheimer’s disease (AD) is a progressive and irreversible disorder of aged brain. AD is characterized by a progressive loss of memory and cognition (Li et al., 2011). There is deterioration in quantity of neurons in different area of the brain, especially in hippocampus that is involved in learning and memory (Herring et al., 2009). Millions of people all over the world are affected by AD, which could potentially manifold in the future. At present there is no definite cure for AD, and current treatments just slow down the progression of this disease and provide symptomatic relief (Robert et al., 2009).

Free radical generation has been associated with cognitive impairment in ICV-STZ model of AD in rats (Veerendra et al., 2003; Sharma and Gupta., 2002). It attack proteins, nucleic acids and lipid membranes and accordingly disrupts the integrity and performance of the cell (Aksenov et al., 2001). ICV-STZ has also been known to impair cholinergic neurotransmission (Sonkusare et al., 2005). The brain tissue contains a lot of unsaturated fatty acids and low level of antioxidants which are especially susceptible for free radical attacks (Gutteridge., 1995). Therefore, antioxidant substances can play an important role in prevention and cure of AD (Ishrat et al., 2009; Khan et al., 2012., Javed et al., 2012).

The inflammation occurs in brain, differs from that found in the other parts of body. The brain lacks pain fibres, making it hard to recognize the occurrence of inflammation and the signs of inflammation. The main component involved in the inflammatory process in AD are considered to be the microglia and the astrocytes and probably to a less extent the neurons, all of which are cellular components who have many critical roles in the homeostasis and function of the brain (Akiyama et al., 2000a, b). However, the particular implications of the inflammatory response for neurodegeneration have not been elucidated. A current hypothesis considers that an extracellular insult to neurons could activate the production of proinflammatory genes like iNOS, (NF-κB) cyclooxygenase-2 (COX-2) (Javed et al., 2012] by astrocytes and microglia. Activation of these genes could affect the normal functions of neuronal cells. Therefore, dysfunction at this core level may lead to abnormalities such as neurofibrillar degeneration and has been identified as a step in the pathway to neuronal degeneration.

STZ is a naturally occurring a glucosamine-nitrosourea compound, generates a cytotoxic product that preferentially destroys β cells in pancreatic islet and produces
diabetes mellitus when it is metabolized. Produced by the bacterium *Streptomycyes achromogenes*, that exhibits broad spectrum antibacterial properties (Vavra et al.1959) ICV injection of STZ in rats produces long-term and progressive learning and memory deficits in rats, oxidative stress, neuro-inflammation, and other biochemical changes that mimic the events in the brain of sporadic Alzheimer disease (AD) patients (Salkovic-Petrisic, 2008; Sharma and Gupta, 2002, javed et al., 2011, Khan et al., 2012). Increased the phosphorylation of tau protein expression in the hippocampus of ICV-STZ rats and some indications of β-amyloid accumulation, suggesting the possibility of developing the SAD hallmarks in this experimental model.

1-piperoylpiperidine (piperine), is a nitrogenous pungent alkaloid presents in the fruits of black pepper (*Piper nigrum*), long pepper (*Piper longum*) and other piper species (family: Piperaceae). These plants are commonly used worldwide as household spices such as food additives and condiments. In addition, they are used as important ingredients for various medicinal purposes in traditional medicine in many Asian countries. Piperine possesses anti-inflammatory (Vaibhav et al., 2012), analgesic effect (Gupta et al., 2000), anticonvulsant (D’Hooge et al., 1996), anti-ulcer (Bai and Xu, 2000) activities, antidepressant (Lee et al., 2005), cognitive enhancing effect (Wattanathorn et al., 2008), cytoprotective and antioxidant activity (Selvendiran et al., 2003). With reference to the effect of piperine on the cognitive function and its antioxidant activity, the effect of piperine on memory impairment and neurodegeneration in Alzheimer’s disease model has been investigated. Therefore, the present study was designed to investigate the neuroprotective role of piperine in inhibition of free radicals and inflammatory enzymes.

**Material and method**

**Chemicals and reagents**

As described in materials and methods, chapter III

**Animals and treatments:**

As described in materials and methods, chapter III
Experimental design

Rats (400 ± 10 g) were divided into six groups of 8 animals

**Group I**: sham operated vehicle treated control (S) group.

**Group II**: ICV-STZ infused and vehicle treated lesion (L) group

**Group III**: ICV-STZ infused and pre-treated with piperine 10 mg/kg body weight (PN10 + L).

**Group IV**: Sham operated and pre-treated with piperine 10 mg/kg body weight (PN10 + S).

Experimental design

![Diagram showing experimental design](image)

**Fig 1.** Experiment design for piperine

**Surgery**

**Intracerebroventricular injection of streptozotocin**

As described in materials and methods, chapter III

**Post-operative care**

As described in materials and methods, chapter III

**Behavioural testing**

As described in materials and methods, chapter III
Biochemical analysis

Tissue preparation
After 21 days of ICV-STZ infusion, the rats were sacrificed and their brains were taken out quickly to dissect the hippocampus and frontal cortex as described in materials and methods chapter III.

Biochemical analysis
The biochemical assays (TBARS, GSH, NO and Protein) are described in materials and methods chapter III.

Hematoxylin and Eosin (H & E) and cresyl violet (CV) stain
The histological studies were done as described in materials and methods chapter III.

Immunohistochemistry of iNOS, NF-κB, COX-2
As described in materials and methods chapter III.

Statistical analysis
As described in materials and methods chapter III

Results:

Effect of piperine on performance in Morris water maze task

Escape latency
Decreased latency showed by all the groups to find the platform from the third to fifth day of experiment. However, L group animals presented a significantly higher latency to find the platform than S group animals, but pre-treatment of piperine has showed a significant improvement in latency in PN+ L group as compared to L group. Slightly decreased in escape latency was observed in PN + S group as compared to sham group. No significant change was observed in piperine pretreated (PN + S) group animal compare to S (Fig 2).

Path length
Significant decreased in path length has been found all group animals from third days to fifth day. L group showed significant increased in path length to find the hidden platform then S group. Pre-treatment with piperine has showed significant improve in
path length in PN+L group from third day to fifth day as compared to L group. Slightly improve in path length was observed in SN+ S group as compared to sham group No significant change was observed in piperine pretreated (PN + S) group animal compare to S (Fig 3)

Fig 2. Effect of piperine on escape escape latency in MWM test in ICV–STZ rats. Values are expressed as mean ± S.E.M *p < 0.05, **p < 0.01 Lesion vs. Sham, #p < 0.05, ##p < 0.01 PN + L vs. Lesion group (L)

Biochemical observations

Piperine pre-treatment reduced TBARS content
The effect of piperine on TBARS content was estimated to show the oxidative damage on membrane lipids in hippocampus and frontal cortex of ICV-STZ–infused rats. A significantly increased (**p < 0.01) and ( *p <0.05) TBARS content was observed in the lesion group animals as compared to S group animals in hippocampus and frontal cortex respectively. This increase in TBARS content was significantly (##p < 0.01) and (#p < 0.05) attenuated in the piperine pre-treated PN + L group animals as compared to lesion group in hippocampus and frontal cortex respectively. The level of TBARS was slightly decreased in PN+S group as compared to sham group. No significant change was observed between the piperine pretreated sham (PN + S) group and sham group

(Fig 4).

Piperine pre-treatment protected GSH content

The protective effect of piperine on GSH content in the hippocampus and frontal cortex is shown in (Fig.5). GSH content was depleted significantly (*p < 0.05) in L group as compared to S group. The decrease in GSH content was protected significantly (#p < 0.05) in PN + L group as compared to L group. The content of GSH was slightly increased in PN+S group as compared to sham group. No significant change was observed between the piperine pretreated sham (PN + S) group and sham group

![Graph showing TBARS levels in hippocampus and frontal cortex](image)

Fig 4. Effects of piperine on TBARS levels in the hippocampus and frontal cortex of ICV–STZ rats. Values are expressed as mean ± S.E.M *p < 0.05 Lesion vs. Sham, #p < 0.01 PN + L vs. Lesion group.
Effect of piperine supplementation on GSH levels in the hippocampus and frontal cortex of ICV–STZ rats. Values are expressed as mean ± S.E.M. *p < 0.05 Lesion vs. Sham, #p < 0.01, PN + L vs. Lesion group.

Effect of piperine on nitrite level

The level of nitrite was significantly (*p < 0.05) increased in the lesion group animals as compared with the sham group animals in the hippocampus and frontal cortex. However, animals pretreated with piperine have significantly protected (## p < 0.01, #p < 0.05) the level of nitrite in hippocampus and frontal cortex when compared with the lesion group animals (Fig 6). The level of nitrite was slightly decreased in PN+S group as compared to sham group. No significant change was observed between the piperine pretreated PN+S and sham group.

Fig 5. Effect of piperine supplementation on GSH levels in the hippocampus and frontal cortex of ICV–STZ rats. Values are expressed as mean ± S.E.M. *p < 0.05 Lesion vs. Sham, #p < 0.01, PN + L vs. Lesion group.

Fig 6. Effect of piperine supplementation on nitrite level in the hippocampus and frontal cortex of ICV-STZ-infused rats. Values are expressed as mean ± S.E.M. *p < 0.05 Lesion vs. Sham, #p < 0.05, **p < 0.01, PN + L vs. Lesion group.
Piperine pre-treatment and H & E staining of CA1 region of hippocampus

Histopathological alteration in neuron after ICV-STZ injection were analysed by H&E staining on the sections of hippocampal CA1 region. The H&E have stained the nucleus in dark blue, and cytoplasmic and intersubcellular substances were stained with varying shades of pink. The loss of pyramidal neurons in CA1 region of hippocampus was not detected in the sham group. On the other hand, lesion group showed the vacuolated tissue architecture and degenerative neurons. PN pre-treatment mitigated hippocampal neuronal alterations in PN+L group as compared to lesion group rats (Fig 7)

Fig 7. Histopathological changes in CA1 region of hippocampus. Sections were stained with H & E. Sham group (B) showed normal pyramidal neuron and black arrows indicate the degenerated pyramidal neurons in lesion group (D). The lesion group pretreated with sesamin shows normal pyramidal neuron staining (F) with less degenerated neurons. Magnification 10x (A, C, E) and 40x (B, D, F).
Effects of piperine (PN) pre-treatment on CV staining of CA1 region of hippocampus

The Section of ICV-STZ infused group showed significant neuronal loss in CA1 region of hippocampus. The intact neurons were almost absent in this area. CV staining of Section of PN+L group animals showed partial loss and shrinkage of neurons in this region. The sham group did not show any pathological changes in this area (Fig 8)
Piperine treatment downregulated the expression expressions of iNOS, NF-κB and COX-2

Immunohistochemical study revealed that ICV-STZ induced number of inflammatory molecules like iNOS, NF-κB, and COX-2. (Fig 9) shows that prominent expression of iNOS, NF-κB, COX-2, were seen in lesion (L) group as compare to sham (S) group. Pretreatment with piperine has decreased the expression of these enzymes in PN+L group as compare to L group. NF-κB is prominent regulator of inflammation and acts as transcriptor regulator of inflammatory proteins like COX-2 and iNOS. The result confirmed that piperine is a strong anti-inflammatory agent.

![Fig.9: Effect of piperine pretreatment on iNOS, NF-κB, COX-2, expression in CA1 region of hippocampus in ICV-STZ-infused rats. Almost negligible staining of iNOS, NF-κB, COX-2 was observed in the S group, whereas over expression of iNOS, NF-κB, COX-2 was observed in the L group showing with black arrow. The lesion group pretreated with piperine PN+L group showed moderate staining of iNOS, NF-κB, COX expression at Magnification of 40X.](image)

**Discussion**

The present study revealed that neuroprotective role of piperine on neurobehavioral oxidative stress, and neuroinflammation in ICV-STZ infused rats. Neuroprotective effect of piperine showed that it is strong antioxidant and anti inflammatory agent which scavenge free radical and down regulate the iNOS, NF-κB, and COX-2
expression corroborating from previous studies (Vaibhav et al., 2012, Shrivastava et al., 2012).

Oxidative stress plays major role in neurodegenerative disease by damaging by damaging biomolecules (lipids, proteins, DNA and RNA as we know that brain is not well equipped with antioxidant enzymes (Javed et al., 2011. Liu et al., 2003). Our results showed that pretreatment with piperine protected the brain injury after ICV-STZ infusion in rats. The antioxidant enhancing role of piperine may contribute to this effect by diminishing oxidative stress These findings are in harmony with the earlier study conducted by our group (Vaibhav et al., 2012, Shrivastava et al., 2012)

In present study the learning and memory deficit in ICV-STZ infused rats was evaluated by using Morris water maze test. In this behavioral test, the animal’s escape from the water reinforces its desire to find the submerged platform quickly, and on subsequent trials, the animal would be able to locate the platform more rapidly. Lesion group (L) showed the significant elevation of escape latency and path length as compare to sham group while, lesion group pretreated with piperine (PN+L) has significantly reduced the time to find the hidden platform in Morris water maze (MWM). Over production production of free radicals cause imbalance between free radicals and antioxidant defences and disturb the redox enviorment of cell and react with protein and nucleic acid to disturb their function or induce lipid peroxidation (LPO) and protein oxidation causing aging and pathogenesis of neurodegenerative diseases (Schulz et al., 2000; Kasapoglu and Ozben,2001, Javed et al.,2012). Therefore scavenging free radicals and preventing lipid and protein peroxidation which is significantly suppress by PN and directly prevent the neuronal cell from oxidative stress and inflammatory response.

We found different oxidative damage biochemical parameters, the important determinant in ICV-STZ induced dementia of Alzheimer’s type (SAD) (Javed et al., 2012, Khan et al., 2012) causes a significant increase in LPO and protein oxidation accompanied by significant depletion in brain GSH. The above circumstances can be reversed by suppressing the overproduction of ROS by endogenous antioxidants, especially by GSH. It is the major endogenous antioxidant in the brain which scavenges free radicals, reduces peroxides and can be conjugated with electrophilic compounds, thereby providing neuronal cells with multiple defences against both ROS and their by-products (Javed et al.,2012). GSH level was found to be low in L group
thus often increase the vulnerability of plasma membranes towards peroxide and other free radicals’ attack. We have observed an elevated level of TBARS accompanied by depleted GSH level in L group and pre-treatment with PN partially attenuates the elevated level of TBARS and depleted level of GSH in hippocampus and frontal cortex.

There was change in the control value of chapter IV, chapter V, chapter VI& chapter VII. In chapter VI&VII we used glass homogenizing tube with Teflon which has maximum rpm of 5000 and the working rpm between 3,000-4,000. At higher rpm there were chances of breaking down of the glass homogenizing tube. In chapter IV&V, Ultra Turex T-25 was used which has rpm 25,000 and the used rpm is between 20,000-25,000, which gives a superb homogenate as compared to glass teflon homogenizer and less cells debris than in glass and teflon homogenizer. Our lab don’t have a fixed temperature, it varies according to the seasons. So the biochemical values were high in summer and low in winter (Chapter IV&V).

It is well documented that iNOS produces NO and NO generate reactive nitrogen species such as peroxynitrite. In healthy neuronal cell, iNOS is not commonly present, but it can be expressed by astrocyte, neurons, and endothelial cells after brain offence where it can initiate the production of high amounts of NO (Vallance and Leiper, 2002). Overproduction of NO may lead to neuronal damage and death. The reaction between NO and super oxide anion generates the cytotoxic compound, peroxynitrite, that leads to neuronal toxicity (Vallance and Leiper, 2002). However in brain inflammation is attributed by number of inducible enzymes like iNOS and COX-2. Therefore it becomes important to evaluate these enzymes in ICV-STZ infused rats with piperine treatment. These inducible isoform of these enzymes COX-2, and inducible iNOS, expression is elevated and leads to the further production of large amount of (NO) and prostaglandins (PGs) which induce neuronal damage (Krakaue T., 2004; Clancy and Abramson, 1995). The present study showed that the up regulation of iNOS and COX-2 in ICV-STZ infused L group and it was restored to normal level in piperine pretreated lesion (PN+L) group animal. These finding suggest that COX-2 and iNOS, is important marker in ICV-STZ induced rats and could be an important target for amelioration of inflammation induced by streptozotocin with piperine. However most of the proinflammatory genes are controlled by NF-κB, which is upregulated in case of ICV-STZ induced animal (Javed et al., 2012). Therefore this
study further needs to investigate the role of piperine in suppression of expression of NF-κB.

NF-κB is a DNA binding hetero or homodimer protein which is a great promoter of transcription. As a downstream transcription factor NF-κB is a key regulator involved in the inducible expression of proinflammatory mediator such as inducible iNOS, COX-2, (Mattson and Camandola., 2001; Surth et al., 2001). When cells are exposed to any stimuli/toxins, NF-κB inhibitors (IkBs) phosphorylated by an upstream IkB kinase (IKK) which leads to it ubiquitination and proteosomal degradation this process liberates p50:p65, which translocate to the nucleus and induces transcription of several genes including iNOS, COX-2. Here we have observed the expression of NF-κB in CA1 region of hippocampus which expression is restored by pre-treatment with piperine in PN+L group. Our result showed that piperine downregulates the expression of NF-κB and reduces the expression of iNOS and COX-2 in CA1 region of hippocampus.