Chapter IV

Effect of hesperidin on neurobehavioral, oxidative stress and lipid profiles in intracerebroventricular streptozotocin induce cognitive impairment in mice
Introduction:

Alzheimer's disease (AD) is the most common cause of dementia in the elderly people and in western industrialised nations. It is the fourth most common cause of death. Around 25 million people worldwide are estimated to be currently affected; however, due to increase in average life span, the occurrence and cost of AD are estimated to quadruple in the next 50 years. AD is characterized by two classical pathological hallmarks in the brain: (i) intracellular neurofibrillary tangles composed of an abnormally phosphorylated form of the tau protein and (ii) extracellular neuritic plaques, composed of a dense amyloid core of β-amyloid peptide (Aβ) surrounded by microglia and dystrophic neurites. It is reported that vascular disorders have been risk factors for Alzheimer's disease and these vascular-based risk factors can be prevented via lifestyle factors such as diet (fish intake, caloric restriction, lowering cholesterol and trans-fat intake) and physical exercise (Kivipelto et al., 2002). A number of studies suggested that physical exercise and diets in high calories such as "good" fats like polyunsaturated fatty acids from fish oil (Freund-Levi et al., 2006; Schaefer et al., 2006) diets low in calories and 'bad' fats (cholesterol and saturated fats) (Simons and Ehehalt, 2002; Wolozin, 2004) may reduce the risk of Alzheimer's disease. These findings are also supported by animal studies in which cognition stimulating environments and caloric restricted diets inhibit neurodegeneration, enhance neurogenesis and improve cognition, and furthermore, the intake of high cholesterol diets influence Alzheimer's disease pathology and cognition in animal models (Hooijmans et al., 2007). The human brain is rich in cholesterol as compared to other organs of the human body. It is estimated that 25% of the total unesterified cholesterol is present in the brain, especially in the myelin sheath and membranes of astrocytes and neurons. Cholesterol plays a key role for the formation of synapses, electrical transmission (Dietschy and Turley, 2004) and serves as a barrier against sodium release (Haines, 2001). Furthermore transgenic mice fed cholesterol rich diets developed increased amounts of neuritic plaques (Hooijmans et al., 2007). In Alzheimer's disease patients, some studies showed high levels of triglycerides in plasma (Razay et al., 2007) while others showed normal or low levels (Mielke et al., 2005). However, apolipoprotein E isoforms are considered to be risk factors for Alzheimer's disease (Siest et al., 2000) and play important role in transport and uptake of triglyceride. These findings indicate that uptake of triglycerides into the brain may be involved in the development of Alzheimer's disease.
The AD pathology is closely related to altered lipid metabolism (Grimm et al., 2006). In neurons, pathogenic Aβ production, low levels of gangliosides and lipid abnormalities may contribute to the pathological conditions in AD (Mutoh et al., 2006). It is also suggested that concentrations of gangliosides in patients with dementia of the Alzheimer's type (DAT) were significantly lower than the concentrations in normal patients (Crino et al., 1989).

Hesperidin is a flavanone glycoside found abundantly in citrus fruits. It has been reported to possess antioxidant (Zhang et al., 2007), analgesic (Galati et al., 1994), hypolipidemic (Montforte et al., 1995), anti-hypertensive and diuretic activity (Galati et al., 1996). Another potential therapeutic application of hesperidin is its anticancer activity mediated through the suppression of cell proliferation (Tanaka et al., 1997a; Tanaka et al., 1997b). The possible hypolipidemic effect of hesperidin is due to its important role in lipid lowering activity. Hesperidin may reduce plasma cholesterol levels by inhibition of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, as well as acyl coenzyme A (Park et al., 2001). Inhibition of these enzymes by hesperidin has been demonstrated in rats fed a high cholesterol diet. These properties of hesperidin have promoted us to study its effect on lipid profiles in animal model of cognitive impairment.

Intracerebroventricular (ICV) administration of subdiabetogenic doses of betacytotoxic drug streptozotocin (STZ) produces long-term and progressive learning and memory deficits in rats, as well as impaired cerebral glucose and energy metabolism that resemble those found in the brain of sporadic Alzheimer's disease (sAD) patients (Lannert and Hoyer, 1998; Prickaerts et al., 1999; Sharma and Gupta, 2001b, 2002). It is also reported that ICV-STZ treated rats showed higher expression of hyperphosphorylated tau protein in the hippocampus, and some reports suggested β-amyloid accumulation in the meningeal capillaries were observed, indicating the possibility of developing sAD pathology in this experimental model, that further supporting the resemblance of this experimental model to human sAD (Salkovic-Petrisic et al., 2006; Grünblatt et al., 2007). Nowadays, it is a well established model to study the sporadic dementia of Alzheimer's type.

**Materials and methods:** As described in section III.
Results

Behavioural observation:

Effect of hesperidin on performance in Morris water maze task:

Latency:

The animals of all groups have improved Morris water maze acquisition performance. S and H200+S group shows decreased latency to find the platform from the second to fifth day of experiment. However, L group animals presented a significantly (p<0.001) higher latency to find the platform than S group, but H100+L and H200+L groups has shown a significant (p<0.01 L vs H100+L; p<0.001 L vs H200+L) improvement as compared to L group (Fig. 1).

In probe trial, L group failed to remember the precise location of the platform, spending significantly (p<0.05) less time in the target quadrant than S group and H200+S-pre-treated group (Fig. 2). The mean percent time spending in the target quadrant was increased significantly by the administration of hesperidin in H100+L and H200+L groups as compared to L group (p < 0.05 L vs H100+L; p<0.01 L vs H200+L).

Biochemical observations

Effect of hesperidin on TBARS content in hippocampus:

The effect of hesperidin on TBARS content was measured to demonstrate the oxidative damage on membrane, in hippocampus of ICV-STZ mice. There was no significant alteration in TBARS content in H200+S group animals in hippocampus while it was elevated significantly (p< 0.01) in L group animals as compared to S group animals. This effect was significantly decreased by hesperidin administration in dose dependent manner. (p<0.05 L vs H100+L; p<0.01 L vs H200+L) [Fig. 3].

Effect of hesperidin on GSH in hippocampus:

The GSH level was significantly (p<0.01) decreased in hippocampus of L group as compared to S group animals. H200+S group animals have exhibited no significant changes in GSH level as compared to S group animals. Pre-treatment of hesperidin has protected the GSH
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level significantly in H100+L and H200+L group animals dose dependently as compared with L group animals (p<0.05 L vs H100+L; p<0.01 L vs H200+L) [Fig. 4].

Effect of hesperidin on acetyl choline esterase activity:
Hesperidin showed significant neuroprotective effect on AchE activity. The activity of AchE was not changed significantly in H200+S group as compared to S group animals and its activity was significantly (p< 0.001) increased in L group as compared to S group animals but it was decreased significantly and dose dependently in H100+L and H200+L group animals as compared to L group animals (p<0.05 L vs H100+L; p<0.01 L vs H200+L) [Fig. 5].

Effect of hesperidin on triglycerides, cholesterol, phospholipids and ganglioside level:
The level of total cholesterol and triglycerides was increased significantly (p<0.001) in L group as compared to S group animals. Hesperidin pretreatment has decreases the level of cholesterol and triglycerides significantly and dose dependently in the H100+L and H200+L group animals as compared with the L group animals (p<0.05 L vs H100+L; p<0.01 L vs H200+L). The level of phospholipids and ganglioside was decreased significantly (p<0.001) in L group as compared to S group animals. Hesperidin pretreatment has maintain the level of phospholipids and ganglioside significantly and dose dependently in H100+L and H200+L group animals as compared with the L group animals (p<0.05 L vs H100+L; p<0.001 L vs H200+L) [Table. 1]
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Fig. 1: Effects of hesperidin pre-treatment on escape latency to locate the hidden platform in Morris water maze task in ICV-STZ infused mice. Values are expressed as mean±S.E.M. (n=10). Average escape latency to find submerged platform was significantly (**p < 0.001) prolonged in L group animals as compared to S group animals. Pre-treatment with H has lessen the time significantly in dose dependent manner to find the hidden platform in H100+L and H200+L group animals as compared with L group animals (##p < 0.01 L vs H100+L, ###p<0.001 L vs H200+L).

Fig. 2: Effects of hesperidin on the mean percentage time spent in the target quadrant in which the platform had previously been located during acquisition in ICV-STZ mice. Values are expressed as mean±S.E.M. of 10 animals. Mean percentage time spent in the target quadrant was decreased significantly (**p<0.01) in L group as compared to S group animals. Hesperidin supplementation significantly increased the time spent in the target quadrant in H100+L and H200+L group in dose dependent manner (##p<0.01 L vs H100+L, ###p<0.01 L vs H200+L).
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Fig. 3: Effect of hesperidin pre-treatment on TBARS content in the hippocampus of ICV-STZ infused mice. Values are expressed as mean ± S.E.M. TBARS content was significantly increased in the L group as compared to S group (**p<0.01 L vs. S group). H pre-treatment significantly decreased TBARS content in dose dependent manner in H 100+L and H 200+L group animals as compared with L group animals (#p<0.05 L vs. H 100+L, ##p<0.01 L vs H 200+L).

Fig. 4: Effect of hesperidin pre-treatment on GSH level in the hippocampus of ICV-STZ infused mice. Values are expressed as mean±S.E.M. The level of GSH was significantly decreased in the L group as compared to S group (**p<0.01 L vs. S group). Hesperidin pre-treatment significantly increased GSH level dose dependently in H 100+L and H 200+L group animals as compared with L group animals (#p<0.05 L vs. H100+L, ##p<0.01 L vs H 200+L).
Fig. 5: Effect of hesperidin pre-treatment on AChE activity in the hippocampus of ICV-STZ infused mice. Values are expressed as mean±S.E.M. AChE activity was significantly increased in L group as compared to S group animals (**p<0.001 L vs. S group). Hesperidin pre-treatment significantly decreased AChE activity in dose dependent manner in H100+L and H200+L group animals compared with L group animals (#p<0.05 L vs. H100+L, ##p<0.01 L vs. H200+L).
The present study was undertaken to demonstrate the effect of hesperidin on memory impairment, oxidative stress, cholinergic dysfunction and lipid alterations on ICV-STZ induced model of memory impairment in mice. The ICV-STZ mice model is an appropriate animal model used for the study of sporadic dementia of Alzheimer’s type (Javed et al., 2011). Morris water maze escape task has been used in this study for spatial learning and memory in animals. This task uses a round pool of water in which a platform is submerged beneath the surface. When placed in the maze the animal’s task is to find the hidden platform. So the time taken by the animals to find the platform over a number of trials is indicating the degree of learning and memory of the animals. The ICV route of administration of STZ showed a persistent deficit in spatial learning and memory as evidenced by no significant reduction in escape latencies in Morris water maze test indicating poorer learning and memory performance, which is consistent with earlier findings (Ishrat et al., 2009a, 2009b; Ishrat et al., 2009c, 2009d; Ishrat et al., 2009e).

<table>
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<th>Parameters</th>
<th>S</th>
<th>L</th>
<th>H100+L</th>
<th>H200+L</th>
<th>H200+S</th>
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<td>Triglycerides (mg/g tissue)</td>
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<td>1.06±0.07</td>
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<td>Ganglioside (mg/g tissue)</td>
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<td>0.30±0.020</td>
<td>0.44±0.038</td>
<td>0.536±0.03</td>
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Table 1: Effect of hesperidin on triglycerides, cholesterol, phospholipids and ganglioside in ICV-STZ infused mice. Hesperidin pretreatment significantly and dose dependently augmented the level of triglycerides, cholesterol, phospholipids and ganglioside in hesperidin pretreated groups as compared to L group animals (***p<0.001 L vs S; #p<0.05 L vs H100+L; ##p<0.01 L vs H200+L; ###p<0.001 L vs H200+L).

Discussion:

The present study was undertaken to demonstrate the effect of hesperidin on memory impairment, oxidative stress, cholinergic dysfunction and lipid alterations on ICV-STZ induced model of memory impairment in mice. The ICV-STZ mice model is an appropriate animal model used for the study of sporadic dementia of Alzheimer’s type (Javed et al., 2011). Morris water maze escape task has been used in this study for spatial learning and memory in animals. This task uses a round pool of water in which a platform is submerged beneath the surface. When placed in the maze the animal’s task is to find the hidden platform. So the time taken by the animals to find the platform over a number of trials is indicating the degree of learning and memory of the animals. The ICV route of administration of STZ showed a persistent deficit in spatial learning and memory as evidenced by no significant reduction in escape latencies in Morris water maze test indicating poorer learning and memory performance, which is consistent with earlier findings (Ishrat et al., 2009a, 2009b; Ishrat et al., 2009c, 2009d; Ishrat et al., 2009e).
Khan et al., 2006; Javed et al., 2011; Tota et al., 2011). However, pretreatment of hesperidin in streptozotocin infused mice showed significantly reduced escape latency in dose dependent manner. On the last day of behavioural test, a probe trial was conducted to test the consolidation of memory by removing the platform from the water pool. In the probe test, streptozotocin infused mice spent less time in the target quadrant (where platform was located) indicating poorer consolidation of memory. Although, hesperidin pretreated group improved memory consolidation significantly and dose dependently as evidenced by increased time spent in target quadrant. It is well documented that hesperidin supplementation is effective in memory dysfunction resulting from nitric oxide mediated memory dysfunction (Gaur et al., 2010).

Free radical induced damage to macromolecules like lipid, protein and nucleic acids are considered as an important factor in the acceleration of aging and age-related neurodegenerative disorders including Alzheimer’s disease (Liu et al., 2001). The antioxidant system uses reduced glutathione (GSH), the most abundant non-protein thiol, which resists free radicals in brain tissue (Dringen, 2000). It eliminates H_2O_2 and organic peroxides by glutathione peroxidase (Meister, 1988). A reduction in the level of GSH may impair H_2O_2 clearance and promotes formation of OH, the most toxic free radical to the brain leading to more oxidant load and consequently oxidative damage. The increase in H_2O_2 induces the peroxidation of polyunsaturated fatty acids leading to the lipid peroxidation (Sun, 1990; Dringen, 2000). Lipid peroxides and hydroperoxides cause secondary injury by further generating relatively more stable and diffusible cytotoxic agents like malondialdehyde (MDA) and intensify oxidative cascade. Increased TBARS content has been reported in AD brains (Arlt et al., 2002). In the present study lipid peroxidation level (in terms of TBARS content) was significantly increased with remarkable learning and memory deficits in L group, which is in agreement with the earlier reports (Javed et al., 2011; Ishrat et al., 2006; Kumar et al., 2003). It was reported that protective effect of hesperidin on lipid peroxidation and glutathione level following brain injury (Gaur et al., 2010). In agreement with this finding, we also found that hesperidin reduced the TBARS content along with increase in glutathione level significantly and dose dependently.

Acetylcholine (Ach) is a neurotransmitter necessary for memory formation and retrieval. Its synthesis depends on the availability of acetyl Co-A, provided by the breakdown of glucose and insulin. Alzheimer’s disease has been linked to a deficiency in the brain Ach (Ishrat et al., 2006). On the other hand, activity of acetylcholine esterase (AchE), a hydrolyzing
enzyme for Ach has been considered as the best marker of cholinergic function. AchE regulates cholinergic nerve and neuromuscular transmission. Increased activity of AchE leads to rapid degradation of Ach that is correlated with cholinergic system abnormalities with intellectual impairment. We observed significantly increased AchE activity in L group, which is consistent with the previous report (Sonkusare et al., 2005). Pretreatment with hesperidin significantly attenuates AchE activity in ICV-STZ infused mice in dose dependent manner.

It is well documented that cholesterol may play an important role in the pathological process of Alzheimer's disease (Hartmann et al., 2001). Epidemiological studies revealed that treatment with cholesterol lowering drug statins, initially prescribed for hypercholesterolemia, decreased the risk of developing Alzheimer's disease (Wolozin et al., 2000; Jick et al., 2000). In laboratory animals, a cholesterol-enriched diet induced amyloid load in the brain (Sparks et al., 1994). It is widely believed that brain cholesterol is related to amyloid metabolism and disruption of cholesterol homeostasis in AD is linked to Aβ pathology (Eckert et al., 2003). In our study, we observed significantly high level of total cholesterol was found in ICV-STZ infused mice as compared to S group animals. Hesperidin significantly attenuated high level of cholesterol in hesperidin pretreated groups dose dependently.

It is reported that Alzheimer's disease patients have high plasma triglycerides level (Razay et al., 2007). However, apolipoprotein E isoforms are thought to be risk factors for Alzheimer's disease (Siest et al., 2000; Hall et al., 2006) and play an important role in the transport and uptake of triglycerides in the brain. This implies that uptake of triglycerides into the brain may be involved in the development of Alzheimer's disease. Fatty acids passively diffuse into the brain or are taken up by carriers (Glatz et al., 2001). Indeed lipids are likely to accumulate with aging in cells and it is also reported that neurons and astrocytes in brain accumulate lipids during aging. The present study showed higher level of triglycerides in the ICV-STZ infused mice and hesperidin higher dose significantly attenuated the level of triglycerides in hepcerin pretreated group.

During aging and neurodegeneration in AD, the physiochemical properties of membranes lipid metabolism undergo significant alterations (Haughey, 2010; Haughey et al., 2010). These changes lead to the imbalances in the proportion of lipids in membranes and/or changed ratios of membrane lipids, which may further leads to the pathogenesis of AD.
Phospholipids are important components of all mammalian cells and play important biological functions, such as formation of lipid bilayers that provide structural integrity, necessary for protein functions and serve as precursors of various second messengers like arachidonic acid and docosahexanoic acid. It is reported that deregulated phospholipids metabolism was reported in neurodegenerative disorder including AD (Adibhatla et al., 2006). Gangliosides are sialic acid-containing glycosphingolipids (GSLs) expressed in the outer leaflet of the plasma membrane of all vertebrate cells but predominantly in nervous system. Gangliosides play key roles in a variety of functions, including serving as antigens, mediators of cell adhesion and modulators of signal transduction and receptors for bacterial toxins (Hakomori, 2003). Earlier reports showed alterations in ganglioside metabolism in AD brain (Svennerholm and Gottfries, 1994). This is manifested as reductions in gangliosides in the majority of brain regions, including the cerebral cortex, hippocampus and frontal white matter, and especially in the frontal cortex and white matter (Kalanj et al., 1991). In neuronal cells, ganglioside and lipid abnormalities as well as pathogenic Aβ production, may lead to the development of pathological conditions of AD (Mutoh et al., 2006). In the present study the level of ganglioside and phospholipids were significantly decreased in ICV-STZ infused mice while hesperidin pretreatment significantly and dose dependently increased the level of ganglioside and phospholipids.