CHAPTER V

Summary and Conclusion
CHAPTER 5

SUMMARY AND CONCLUSION

The work embodied in this thesis was carried out to see in depth the modulation of a metabolic functions during normal aging in animals together with their modulation and changes in diabetic animals. It has recently been suggested that diabetes, a condition of insulin deficiency can be said to be a hastened process of aging. In the present work two compounds *Trigonella foenum graecum* and estradiol were tested for their antidiabetic and antiaging properties respectively. Close correlations have recently been shown among the late onset complications encountered in diabetes and aging linked to neurobiological disorders. Aging in females and males is considered as the end of natural protection against age related diseases like osteoporosis, coronary heart disease, diabetes, Alzheimer’s disease and Parkinson’s disease, dementia, cognitive dysfunction and hypernatremia. Besides the sex hormones other hormonal changes are also known to occur during aging and many common problems encountered in the aging process can be related to neuroendocrine phenomena. *Diabetes mellitus* is associated with moderate cognitive deficits and neurophysiologic and structural changes in the brain, a condition that may be referred to as diabetic encephalopathy, diabetes increases the risk of dementia especially in the elderly. The current view is that the diabetic brain features many symptoms that are best described as accelerated brain aging.

ACCELERATED AGING OF THE BRAIN IN DIABETES

The observation that the effects of diabetes on the brain appear to be most pronounced in the elderly should be taken into consideration when the mechanisms that underlie diabetic encephalopathy are explored. The aging brain is possibly more sensitive to the effects of diabetes. Alternatively, the pathogenic processes of aging and diabetes might interact, leading to an accelerated cognitive decline in the elderly.

Like aging, diabetes is associated with an impairment of neuronal Ca$^{2+}$ homeostasis. Obviously, the relative contribution of ischaemia, oxidative stress, advanced glycation end products formation and disturbances of neuronal Ca$^{2+}$ homeostasis is different in brain aging.
and the development of diabetic encephalopathy; however, the similarities are evident and are likely to explain part of the increased susceptibility of elderly patients to the effects of diabetes on the brain. Experimental evidence for an interaction between diabetes and aging is provided by the aforementioned observation that streptozotocin diabetes produces more severe deficits in learning and synaptic plasticity in aged (24 months) than in young adult Wistar rats.

With regard to the mechanisms by which they damage the body, aging and diabetes have much in common. Both of them involve: increased free radical activity due to loss of antioxidants; damage to structural proteins caused by cross-linking; damage to genes by free radicals and by sugar aldehydes; accumulation of bulky molecular debris, including amyloid and lipofuscin.

**DIABETES AND TRIGONELLA FOENUM GRAECUM TREATMENT**

The term diabetes mellitus describes a metabolic disorder of multiple aetiology, characterized by chronic hyperglycemia with disturbances of carbohydrate, fat and protein metabolism, resulting from defects in either insulin secretion or insulin action or both. A number of Indian herbal preparations have been used in the treatment of diabetes from ancient times. *Trigonella foenum graecum* (Fenugreek) appears to have potential anti-diabetic effects by stimulating or regenerating pancreatic beta cells, its extra pancreatic effects may be important. *Trigonella foenum graecum* seeds powder (TSP) administration for three weeks to alloxan diabetic rats stabilizes glucose homeostasis in brain by normalizing the glucose and membrane linked and antioxidant enzymes. *Trigonella* seed supplementation in the diet also normalizes the free radical metabolism in alloxan diabetic rats.

**AGING AND ESTRADIOL TREATMENT**

Estrogen acts through its receptor and regulates the development and the function of various human organ system including reproductive, skeletal and nervous system, it is also involved directly in numerous pathological and aging processes such as Alzheimer disease, osteoporosis, cardiovascular disease, mental disorder and carcinogenesis.

Aging is associated with a decline in metabolic function, and one of the main illustrations of this metabolic decline is the development of insulin resistance. Although the
mechanism underlying the development of insulin resistance with advanced age remains unclear, in the case of females, loss of gonadal function seems to determine the start of this period of metabolic decline. In addition, insulin resistance in aging is associated with metabolic syndrome, which is associated with increased incidences of depression, neurodegenerative diseases, and memory or cognitive dysfunction. Therefore, the increased incidences of neurodegenerative diseases in postmenopausal females seem to be clinically associated with aging, loss of gonadal function, and development of insulin resistance.

**OBJECTIVES**

1. A synaptosome is an isolated synapse of a neuron. Synaptosomes are formed from the phospholipid layer of the cell membrane carrying out synaptic transmission, they contain the molecular machinery necessary for the uptake, storage, and release of neurotransmitters and are relatively easy to prepare in the laboratory. Whole brain synaptosomes were used as *in vitro* models to evaluate various effects on diabetic and glucose homeostasis and insulin signaling on brain, in diabetes and aging.

2. The objective of the study was to correlate and study the biochemical, histochemical and molecular processes taking place in experimental diabetes and normal aging.

3. The two physiologically different condition, diabetes and aging are taken and various neuronal/membrane markers, physiological, biochemical, histochemical, structural and molecular, were studied with their reversal using an antidiabetic compound, *Trigonella foenum graecum* and the hormone estradiol (E2).

4. The procedures used were that female animals of 3, 12, 24 months were taken together with estradiol groups of the same age, and diabetic animals treated with *Trigonella* and insulin.

5. General parameters and other neuronal/membrane parameters were studied, physiological (body weight, hormone levels, glucose and protein levels) biochemical (membrane bound (Na⁺K⁺ ATPase, Ca⁺⁺ ATPase, Monoamine oxidase), antioxidant enzymes (Superoxide dismutase, Glutathione S-transferases), lipid peroxidation), structural changes (degree of membrane fluidity, intrasynaptosomal calcium levels), histochemical changes (neurolipofuscin accumulation, immunohistochemistry of Glucose transporter-4 (GLUT4), and molecular changes (GLUT4 mRNA expression,
Western blotting, DNA laddering) including insulin levels in both diabetic and aging were measured.

6. Results from both physiologically induced conditions are discussed in relation to each other and separately to elucidate the similarities of the two conditions, diabetes and aging.

**THE RELEVANCE AND EXPECTED OUTCOME OF THE PROPOSED STUDY**

This study presents and compares biochemical, physiological, molecular and pathological data from neuronal tissue of aging and hormone treated control and diabetic animals to arrive at the similarities among the two naturally occurring physiological conditions. Animal models can make a substantial contribution to understanding of the pathogenesis, which share many features with mechanism underlying brain aging. By studying the pathogenesis, targets for pharmacology can be identified, finally leading to delay or prevention of these complications. Antiaging strategies using hormone therapy, was carried out for reversal of aging effects. Neuronal markers have been presented in this study and similarities in changes were seen among the aging, diabetes and hormone treated (estrogen and insulin) brains from these animals. A close correlation was observed in parameters like oxidative stress, enzyme changes, and pathological changes like lipofuscin accumulation in aging and diabetic brain.

**EXPERIMENTAL DIABETES**

**1. EFFECT OF ANTIDIABETIC COMPOUNDS ON GENERAL PARAMETERS**

There is significant decrease in body weight of diabetic animals after three weeks when compared with control. Alloxan-diabetic rats were characterized by four-fold increase in the blood glucose levels. The three weeks of treatment with insulin and Trigonella resulted in a marked reduction in hyperglycemia in the diabetic rats. Hyperglycemia has been shown to generate free radicals from autooxidation of glucose, formation of advanced glycation end products and increased polyol pathway, with concomitant increase in cellular lipid peroxidation and damage of membrane in diabetes. There is significant decrease in insulin levels in diabetic groups when compared with control. Trigonella treatment restored the insulin levels close to normal in diabetic rats.
Summary and Conclusion

2. EFFECT OF ANTIDIABETIC COMPOUNDS ON ENZYMES

MEMBRANE BOUND ENZYMES
There was a decrease in the activities of Na\(^+\)K\(^+\) ATPase and Ca\(^{2+}\)ATPase in synaptosomal fractions of whole brain of diabetic rats after 21 days of diabetes induction. Activity of catecholamine degrading enzyme, monoamine oxidase (MAO), showed a significant increase in the synaptosomal fractions of whole brain in diabetic animals. Results show that diabetes affect MAO, causing a marked increase in the enzyme activity in both synaptosomes and supernatant fraction of brain in diabetic rats. Reactive oxygen substances affect membrane linked enzyme activity through modification of membrane fluidity because the activity of most membrane bound enzymes is regulated by the physiochemical state of their lipid environment. Treatment of diabetic rats with insulin and *Trigonella* separately restored the alterations in the enzyme activity.

ANTIOXIDANT ENZYMES
The activities of superoxide dismutase (SOD) and glutathione S-transferases (GST) were measured in brain cytosolic fractions of diabetic and diabetic rats with insulin and *Trigonella*. Superoxide dismutase (SOD) is one of the most important enzyme in the anti-oxidant defense system. A significant decrease was observed in the activities of superoxide dismutase and glutathione S-transferases in the brains of diabetic rats. Treatment of diabetic rats with insulin and *Trigonella* separately restored the alterations in the antioxidant enzyme activities. *Trigonella* probably improved antioxidant status in diabetes by decreasing oxidative stress.

3. EFFECT OF ANTIDIABETIC COMPOUNDS ON LIPID PEROXIDATION
There was a significant increase in peroxidative damage as measured in the formation of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) which are produced by lipid peroxidation in cells in the diabetic state. Treatment with insulin and *Trigonella* brought malondialdehyde levels close to control values. The increased accumulation of this byproducts strongly reflects alloxan induced oxidative damage. Inhibition of these byproducts by insulin administration and *Trigonella* strongly suggested their anti-lipidperoxidative abilities. After that treatment there was significant reversal near to the normal value.
4. EFFECT OF ANTIDIABETIC COMPOUNDS ON MEMBRANE FLUIDITY
The fluidity in the synaptosomal fractions from the brain as observed, by polarization and anisotropy measurement on membranes from diabetic groups indicate decreased fluidity in the lipid structure. The decrease in membrane fluidity of diabetic brain could be due the peroxidation of membrane phospholipids through free radicals, generated by persistent hyperglycemia. With insulin and Trigonella treatment there was a reversal in membrane fluidity close to normal. Trigonella probably restored fluidity in diabetes by decreasing oxidative stress.

5. EFFECT OF ANTIDIABETIC COMPOUNDS ON NEUROLIPOFUSCIN ACCUMULATION
The results showed that lipofuscin deposition was increased with diabetes in all three brain regions i.e. cerebral hemispheres, cerebellum and brain stem. After the insulin and Trigonella treatment to diabetic animals, there was decrease in lipofuscin deposition in neurons and also an increase in the number of neurons without lipofuscin in the three different brain regions when compared with the respected control age groups rat. This may be due to the decreased level of lipid peroxidation and decreased level of oxidative stress in the diabetic brain regions with antidiabetic compound treatment.

6. EFFECT OF ANTIDIABETIC COMPOUNDS ON CALCIUM HOMEOSTASIS
In the present study the synaptosomal calcium (Ca$^{2+}$) increased in diabetes due to oxidative stress and membrane damage. An increase in synaptosomal Ca$^{2+}$ in the diabetic tissue could be due to excessive lipid peroxidation and a decrease in membrane fluidity. Chronic hyperglycemia induced a number of changes in the neurochemical profile possibly linked to osmolarity regulation essential for the maintenance of cellular homeostasis. Trigonella and insulin treatment restored the altered synaptosomal Ca$^{2+}$ levels to controls. TSP treatment decreased the oxidative stress and lipid peroxidation levels. Trigonella probably improved calcium homeostasis in diabetes by decreasing oxidative stress.
7. EFFECT OF ANTIDIABETIC COMPOUNDS ON DNA DEGRADATION
Oxidative stress is thought to play an important role in the pathogenesis and complications of diabetes and increased risks of oxidative DNA degradation/fragmentation. In this study, degradation of genomic DNA, a marker of apoptosis, was determined by using DNA laddering method. Oxidative DNA degradation was increased in the cerebral cortex of diabetic animals as compared to the age matched controls. The diabetes state is associated with an increase in the rate of H₂O₂ production and decreased antioxidant system, leading to the accumulation of H₂O₂, causing DNA degradation. Thus the measurement of oxidative DNA degradation in diabetic state by using laddering method is a suitable marker for the evaluation of systemic oxidative stress in diabetic patients. Furthermore, the treatment of diabetic rats with insulin and *Trigonella* prevented genomic DNA fragmentation.

8. EFFECT OF ANTIDIABETIC COMPOUNDS ON GLUT4 TRANSLOCATION
The expression and distribution of glucose transporter-4 (GLUT4) was measured by RT PCR, immunoblotting and immunohistochemical techniques in the membrane fractions of cerebral cortex of control, diabetic and diabetic rats after 21 days of treatment with insulin and *Trigonella*. There was a marked decrease in the GLUT4 protein levels in the membrane fractions of the cerebral cortex of diabetic animals. Treatment with antidiabetic compounds for 21 days restored the GLUT4 levels to those of control values. The reversal pattern was further assessed by immunohistochemical study of cerebral cortex of control, diabetic and antidiabetic treated rats clearly showing the efficacy of the *Trigonella* therapy equivalent with insulin treatment.

AGING
The second objective of this study was to study and observe changes occurring in the brains of aging female rats and to see whether these changes are reverted to normal levels after exogenous administration of estradiol and the extent of changes in young, adult and old rats. To find out whether administration of estradiol at different ages of the animals prevents the delay of biochemical and physiological alterations known to be associated with aging.
1. EFFECT OF ESTRADIOL ON GENERAL PHYSIOLOGICAL PARAMETERS IN AGING

There is significant increase in body weight of animals with aging with compared with 3 months control groups. In 24 months aging there was significant decrease in body weight with estradiol treatment. It can be due to anti-obesity action of hormone that reduced fat deposition in 24 months old rats. The protein concentrations in brain were increasing with age, after the hormonal treatment the protein level further increases in tissues, because of this reason the tissue weight increases in hormone treated rats. The glucose levels in blood increases with increasing age. After the treatment with estradiol the concentration increased, this may be due to an increase uptake of glucose by the cells. There was an increase in insulin levels with aging with estradiol treatment there was a reversal in insulin levels to normal values.

2. EFFECT OF ESTRADIOL ON ENZYMES ACTIVITIES IN AGING

MEMBRANE BOUND ENZYMES

In the present study the Na\(^+\) K\(^+\) ATPase and Ca\(^{2+}\) ATPase activities were reduced, MAO activity increased with age and after the estradiol treatment the activity was restored. The decreased activity may be due to loss of neurons in the nerve tissue or decreased synthesis of protein/mRNA in tissues, resulting in reduced conduction/impulse in the nerve tissue. After the hormone treatment the activity was increased, this may be due to the prevention in the brain from oxidative damage and decreased in lipid peroxidation levels.

ANTIOXIDANT ENZYMES

The activities of superoxide dismutase and glutathione S-transferases were measured in young and old control and estradiol treated rats of different age groups in the brain. The activities of antioxidant enzymes were decreased with aging and after the estradiol treatments the activities were increased in all the age groups. A decline in normal antioxidant defense mechanisms is postulated to be a causative factor in aging-related declines in metabolic function. After the treatment with estradiol the activity increased. This increase may be the
increased synthesis of protein and its mRNA also the free radicals production in cells is decreased.

3. EFFECT OF ESTRADIOL ON LIPID PEROXIDATION IN AGING
The increased production of peroxidants with aging might be derived from the membrane damage and free radical-mediated injury to tissues. The lipid peroxidation level was increases with age. Estradiol may reduce the free radical production thereby reducing the lipid peroxidation production in brain and prevents from free radical-mediated injury. With aging there was a significant increase in lipid peroxidation (malondialdehyde and 4-Hydroxynonenal) and it is more in 24 months old rats when compared with 3 months control groups. Aging rats treated with estradiol reversed the malondialdehyde and 4-Hydroxynonenal levels to the normal levels. Inhibition of these by-products by estradiol administration suggests its anti-lipidperoxidative abilities.

4. EFFECT OF ESTRADIOL ON MEMBRANE FLUIDITY IN AGING
Polarization and anisotropy measurement on synaptosomal membranes from different age groups indicate decreased fluidity in the lipid structure. The decrease in membrane fluidity in aging brain synaptosomes could be due the peroxidation of membrane phospholipids through free radicals, which is generated by persistent oxidative stress. The treatment of aging animals with estradiol reverts the membrane fluidity to normal level when compared with respective control.

5. EFFECT OF ESTRADIOL ON NEUROLIPOFUSCIN ACCUMULATION IN AGING
In the present study, an age related increase in the deposition of lipofuscin in aged animals was observed. Lipofuscin deposition was increased with aging in all three brain regions i.e. cerebral hemispheres, cerebellum and brain stem. The increased amount of lipid peroxides is one of the factors for the accumulation of lipofuscin in brains of aged rats. After the treatment with estradiol to 12 and 24 months old rats a decreased lipofuscin accumulation
and less dense lipofuscin deposition in all the brain regions. This may be due to decreased level of lipid peroxidation and oxidative stress in the brain cells.

6. EFFECT OF ESTRADIOL ON CALCIUM HOMEOSTASIS IN AGING
In the present study, the levels of synaptosomal Ca$^{2+}$ were significantly increased in aged rats. This increase in intracellular calcium may be due to increased production of free radicals and loss of mitochondrial membrane integrity. The results show that the prevention of dysregulation of intracellular Ca$^{2+}$ homeostasis by estradiol is advantageous in aged rats. Possibly, estradiol may prevent the inhibition of the Ca$^{2+}$ ATPase pump and selective Ca$^{2+}$ permeability, by scavenging reactive oxygen radicals.

7. EFFECT OF ESTRADIOL ON DNA DEGRADATION IN AGING
In the present study genomic DNA isolated from young 3 months control animals showed no evidence of DNA fragmentation. In contrast, with aging there was increased in the banding pattern, which is a characteristic of DNA fragmentation and apoptosis. Furthermore, the treatment of aging rats with estradiol treatment prevented genomic DNA fragmentation. This may be due to decreased oxidative stress and increased antioxidant status in the brain cells.

8. EFFECT OF ESTRADIOL ON GLUCOSE TRANSPORTER-4 (GLUT4) EXPRESSION IN AGING
The expression and distribution of glucose transporter-4 (GLUT4) was measured by RT PCR, immunoblotting and immunohistochemical techniques in the membrane fractions of cerebral cortex of aging and estradiol treated rats. With the aging there was a marked decrease in the GLUT4 content in the membrane. Estradiol treatment of aging rats resulted in normalization of GLUT4 levels in the brain membrane fractions. GLUT4 distribution in cerebral cortex of aging and estradiol treated rats was also checked by immunohistochemistry and analyzed by light microscopy. GLUT4 was localized predominantly on the membrane in
Summary and Conclusion

control rat brain. Treatment with estradiol corrected the alterations in the distribution of GLUT4 by decreased oxidative damage and increased antioxidant status.

Present results shows that there was a similar pattern of increased lipid peroxidation, neurolipofuscin, DNA degradation and monoamine oxidase activity and a decrease in membrane fluidity, Na\(^+\) K\(^+\) ATPse, Ca\(^{2+}\) ATPase, superoxide dismutase and glutathione S-transferases activities, glucose transporter-4 (GLUT4) in both aging and diabetes. The insulinotropic property of 4-hydroxisoleucine, an amino acid extracted from *Trigonella foenum graecum* suggested the involvement of insulin secretion modulation in its therapeutic action. *Trigonella foenum graecum* was found to be an effective treatment in stabilizing and normalizing the membrane functions; therefore this therapy can be considered an alternative to be explored further as a means of diabetic control. Estradiol treatment also helped to reverse the age related changes studied, to normal levels, elucidating an anti-aging and neuroprotective action. Estradiol’s beneficial effects seemed to arise from its antilipofuscin, antioxidant, antilipidperoxidative, and anti-aging action. It can therefore be concluded that the long-term hormone treatment at much lower doses than hormone replacement therapy levels, used in the present experiment contributes to the decreased oxidative stress, and other neurodegenerative factors, which increased with aging. The results of this study will be useful for pharmacological modification of the aging process and applying new strategies for control of age related disorders including metabolic syndrome.
### SUMMARY OF RESULTS

<table>
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<tr>
<th>Parameters changes</th>
<th>Aging</th>
<th>Estradiol Treatment</th>
<th>Diabetes</th>
<th>Trigonella treatment</th>
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<td>(b) Na⁺ K⁺ ATPase</td>
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↓ Decreased levels  ↑ Increased levels
TSP probably increases insulin action/or secretion and decrease free radicals.

- **Hyperglycemia**
- **Neuronal changes**
  - Reduced uptake of glucose
  - Increased Oxidative Stress
  - Altered Membrane Effects
  - Decreased serum insulin
  - Increased production of AGEs
  - Increased Free Radicals
    - Increased MDA and 4HNE
    - Lipofuscin
      - Decreased
      - Reversed to near control levels

**Trigonella foenum greacum seed powder (TSP)**

- Decrease ROS
- Membrane fluidity
- GLUT 4

**Proposed Mechanism of action of TSP treatment on neuronal changes in diabetic rat**
Estradiol Probably Decrease free radicals

Aging

Increased Free Radicals

Membrane damages

Increased Oxidative Stress

Enzymes Metabolites

DNA degradation

Altered Membrane Effects

\[ \text{Ca}^{2+} \text{ ATPase Fluidity} \]

Altered Metabolic Changes

Increased production of AGEs

Neuronal degeneration

Reversed to Control Levels

Estradiol

PROPOSED MECHANISM OF ACTION OF ESTRADIOL TREATMENT ON NEURONAL CHANGES IN AGING RATS