CHAPTER 4A
TIME-RESPONSE STUDIES ON DEVELOPMENT OF
COGNITIVE DEFICITS IN AN EXPERIMENTAL MODEL OF
INSULIN RESISTANCE

4.1.1. INTRODUCTION

Insulin plays a critical role in glucose utilization and control of metabolism in peripheral tissues. It is readily transported into the central nervous system across the blood–brain barrier by a saturable, receptor-mediated process (Cholerton et al., 2011). Insulin receptors are located in the synapses of both astrocytes and neurons (Abbott et al., 1999). The insulin receptor and downstream signaling molecules including insulin receptor substrate are expressed throughout the peripheral and central nervous systems.

The brain content of insulin is normally fine tuned to circulating insulin concentrations (Wallum et al., 1987) but peripheral hyperinsulinemia down-regulates blood–brain barrier insulin receptors and reduces insulin transport into the brain and thus leads to decreased CSF: plasma insulin ratio (Schwartz et al., 1990).

There is an extensive literature showing that insulin has an important role in physiological hippocampal memory processes (van der Heide et al., 2006; McNay et al., 2010). Neuronal insulin resistance has been suggested to be directly linked to the development of neurodegenerative diseases such as Alzheimer’s disease (Kim and Feldman, 2012). Defective brain insulin signaling is associated with decreased cognitive ability and development of dementia, leading to the qualification of Alzheimer’s disease as ‘an insulin-resistant brain state’, sometimes referred to as diabetes mellitus type3 (Banks et al., 2012).

Abundant consumption of fructose is an important contributor in triggering an explosive surge in metabolic syndrome (Gerrits and Tsalikian, 1993). Studies have shown that high-fructose consumption causes
hyperinsulinemia, hypertriglyceridemia, hepatic steatosis as well as endothelial dysfunction in rats (Hwang et al., 1987; Tran et al., 2009). Fructose is preferentially metabolized by the liver into lipids and produces large increases in plasma triglyceride (TG) concentrations (Basciano et al., 2005; Havel, 2005). TG can penetrate the blood brain barrier and can impair memory by producing hippocampal insulin resistance (Ross et al., 2009). Injection of TGs directly into the brain ventricles has been shown to impair memory (Farr et al., 2008). Evidence is accumulating that neuronal cells can metabolize fructose (Funari et al., 2007) and that fructose feeding increases the expression of fructose sensitive glucose transporters (GLUT 5) in the hippocampus (Shu et al., 2006).

However, an overview of literature provides a variable opinion regarding the fructose concentration and duration of fructose feeding required to induce insulin resistance and resultant protracted cognitive function. Previous studies reveal that acute ingestion of fructose leads to improved memory (Messier et al., 2007). However, long term access to fructose induces variable effects on learning and memory (Ross et al., 2009; Yin et al., 2014).

Thus the present study aimed to construct a time-response curve of appearance of insulin resistance and prediction of memory deficits by feeding 15% fructose solution to rats over a period of 24 weeks. Further, an attempt was made to draw correlations between indices of insulin resistance and behavioral markers of memory at various time points throughout the study period.
4.1.2. Material and Methods

4.1.2.1. Animals and experimental design:

Male rats (140–160 g) bred in Central Animal House facility of Panjab University were used. The animals were housed under standard laboratory conditions, maintained on a 12:12 h light:dark cycle and had free access to food (Ashirwad Industries, Mohali, India) and water. Animals were acclimatized to laboratory conditions before all the behavioral tests. All experiments were carried out between 0900 and 1700 h. The experimental protocols were approved by the Institutional Animal Ethics Committee (IAEC/175/UIPS/13 dated 30/8/11) of Panjab University and performed in accordance with the guidelines of Committee for Control and Supervision of Experimentation on Animals (CPCSEA), Government of India, on animal experimentation.

The rats were randomly assigned to fourteen different groups viz Group-1-7 comprised of control rats for each time point, being fed with normal drinking water and chow feed whereas Group-8-14 rats received 15% w/v D-fructose solution as a substitute for drinking water along with fed with chow feed for 7th, 10th, 14th, 16th, 18th, 20th, 22nd and 24th weeks respectively for development of insulin resistance and associated memory impairment.

Blood was collected at different time point i.e. after 7th, 10th, 14th, 16th, 18th, 20th, 22nd and 24th weeks to assessment of physiological parameters. Rats were sacrificed by cervical dislocation and brains were harvested, cleaned with chilled saline and stored at -80°C till further analysis.

4.1.2.2. Drugs and reagents

D-fructose was purchased from Thermo Fisher Scientific India Pvt. Ltd., Mumbai. Tumor necrosis factor-α (TNF- α), Tumor Growth Factor (TGF-β) and Interleukin-1 beta (IL-1β) ELISA kits were purchased from R&D Systems, Minneapolis, MN, USA. Insulin and homocysteine ELISA kits were purchased from DRG, Marburg, Germany and Wkea Med Supplies Corp, China respectively. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and caspase-3 ELISA kits were purchased from Imegenex, San
Diego, USA and Biovision, USA respectively. All other chemicals used for biochemical measurements were of analytical grade.

Experimental Design and treatment protocol

4.1.2.3. Assessment of physiological parameters-

i. **Body weight, food and water consumption and blood pressure**

Body weight, food, water intake was monitored every week for 24 weeks and blood pressure was monitored on 24th week and compared with respective control rats.

ii. **Measurement of plasma glucose**

Plasma glucose was measured by GOD-POD method using diagnostic kits (ERBA Diagnostics, Mannheim GmbH, Germany) on 7th, 10th, 14th, 16th, 18th, 20th, 22nd and 24th week.

**Method for glucose estimation:**

Principle:

\[
\text{Glucose + O}_2 + \text{H}_2\text{O} \rightarrow \text{Gluconic acid + H}_2\text{O}_2 \\
\text{H}_2\text{O}_2 + 4\text{HBA} + 4\text{AAP} \rightarrow \text{Quinoneimine dye + 2H}_2\text{O}
\]
4AAP: 4-Aminoantipyrine, 4HBA: 4-Hydroxy Benzoic acid

The intensity of the pink colour formed is proportional to glucose concentration and it was measured spectrophotometrically at 510 nm.

**Reagents 1: Glucose reagent**

<table>
<thead>
<tr>
<th>Glucose oxidase</th>
<th>20000IU/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peroxidase</td>
<td>3250IU/L</td>
</tr>
<tr>
<td>4-Aminoantipyrine</td>
<td>0.52m mol/L</td>
</tr>
<tr>
<td>4-Hydroxybenzoic acid</td>
<td>10 m mol/L</td>
</tr>
<tr>
<td>Phosphate buffer</td>
<td>110 mmol/L</td>
</tr>
</tbody>
</table>

**Reagent 2: Glucose standard (100 mg/dl)**

*Preparation of working reagent:* The vial was allowed to attain the room temperature (15-30°C). The contents of each vial were dissolved using Glucose diluents with specific clearing agent. The final volume was made to 200 ml or 500 ml depending on the pack size and transferred into a clean dry amber colored bottle.

**Assay procedure:**

\[
\text{Glucose (mg/dl)} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \text{Concentration of standard (mg/dl)}
\]

<table>
<thead>
<tr>
<th>Pipette into tubes marked</th>
<th>Blank</th>
<th>Standard</th>
<th>Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Working reagent</td>
<td>1000μl</td>
<td>1000μl</td>
<td>1000μl</td>
</tr>
<tr>
<td>Distilled water</td>
<td>10μl</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Standard</td>
<td>-</td>
<td>10μl</td>
<td>-</td>
</tr>
<tr>
<td>Test</td>
<td>-</td>
<td>-</td>
<td>10μl</td>
</tr>
</tbody>
</table>
All the reagents were mixed well and incubated for 15 minutes at 37°C. The absorbance of the standard and sample was read against reagent blank at 505 nm.

Results were expressed mg/dl.

iii. **Measurement of Plasma insulin**

**Insulin ELISA**

Plasma insulin was measured by using ELISA kits (DRG, Marburg, Germany) on 7th, 10th, 14th, 16th, 18th, 20th, 22nd and 24th week.

**Principle:**

Rat insulin in the sample was bound to the rat anti-insulin antibody coated on the microplate well. Horse radish peroxidase (POD)-conjugated anti-insulin antibody was then bound to the rat anti-insulin antibody/rat insulin complex immobilized to the microplate well. The bound POD conjugate in the microplate well was detected by the addition of the 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution. The insulin concentration was determined via interpolation using standard curve generated by plotting absorbance versus the corresponding concentration of rat insulin standard.

**Assay Procedure:**

1. The antibody-coated microplate was removed from the sealed foil pouch and equilibrated to room temperature. The microplate was affixed to the supporting frame.

2. In each well, 95 μL of sample diluent was dispensed.

3. 5 μL samples (or 0, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2 and 6.4 ng/mL working rat insulin standards) was pipetted into the wells.

4. The microplate was covered with the plastic microplate cover and incubated for 2 hours at 4°C.

5. The microplate was washed five times using 300 μL of wash buffer per well. After each wash, the remaining solution was removed by inverting and tapping the plate firmly on clean paper towel.

6. 100 μL per well of anti-insulin enzyme conjugate was dispensed.
7. The microplate was covered with the plastic microplate cover and incubated for 30 minutes at room temperature.

8. The microplate was washed seven times using 300 μL of wash buffer per well. After each wash, the remaining solution was removed by inverting and tapping the plate firmly on clean paper towel.

9. 100 μL per well of enzyme substrate solution was immediately dispensed and allowed to react for 40 minutes at room temperature. During the enzyme reaction, avoid exposing the microplate to light.

10. The enzyme reaction was stopped by adding 100 μL per well of enzyme reaction stop solution.

11. The absorbance of each well was read using a microplate reader set to 450 nm within 30 minutes. The concentrations of insulin were calculated from plotted standard curves.

iv. **Measurement of Glycosylated hemoglobin (HbA1c)**

Glycosylated hemoglobin was measured by cation exchange resin method by using diagnostic kits (Excel Diagnostics, Hyderabad) on 7th, 10th, 14th, 16th, 18th, 20th, 22nd and 24th week.

It is normal adult hemoglobin (HbA1) which is covalently bonded to a glucose molecule. Glycosylated hemoglobin (GHB) concentration is dependent on the average blood glucose concentration. It is formed progressively and irreversibly over a period of time and is stable till the life of the RBC. A single glucose determination gives a value which is true only at the time the blood sample is drawn. GHB on the other hand is unaffected by diet, insulin or exercise on the day of testing and thus reflects the average glucose level over the last several weeks. Hence, it reflects on the long term metabolic control of glucose in individuals. GHB is now widely recognised as an important test for the diagnosis of Diabetes mellitus and is a reliable indicator of the efficacy of therapy. There are several acceptable methods of GHB measurement like electrophoresis, ion-exchange chromatography, affinity chromatography, HPLC and colorimetry. HbA1c level was measured
by cation exchange resin method (Excel Diagnostics, Hyderabad) and GHb kit based upon the property of non-glycosylated hemoglobin to bind with a weak cation exchange resin leaving GHb free in the supernatant.

**Principle:**

Whole blood is mixed with lysing reagent to prepare a hemolysate. This is then mixed with a weakly binding cation exchange resin. The non-glycosylated hemoglobin binds to the resin leaving GHb free in the supernatant. The GHb percentage is determined by measuring the absorbance of the GHb fraction and of the total Hb.

**Reagent preparation:**

Dissolve the control with 0.5 ml of deionized water by inverting / swirling. Do not shake vigorously. Reconstituted control is stable for 3 days at 2-8°C or 21 days at -20°C.

**Procedure:**

Assay temperature : 23±1°C or 30±1°C; Wave length : 415 nm

**Step I - Hemolysate preparation**

1. Pipette 0.25 ml of lysing reagent (2) in a test tube.
2. Add to it 0.05 ml of well mixed sample/control.
3. Mix well and allow to stand at room temperature for 5 minutes.

**Step II - GHb separation and assay**

1. Bring a Resin Tube (1) to assay temperature by incubating the tube in water bath.
2. Add to it 0.1 ml of hemolysate (from step 1)
3. Position a Resin Separator in the tube, so that the rubber sleeve is approximately 3 cms, above the resin level.
4. Mix the contents on vortex mixer continuously for 5 minutes
5. Allow the Resin to settle at assay temperature for 5 minutes push down the Resin separator in the tube until the Resin is firmly packed.
6. Pour the supernatant directly into a cuvette and measure the absorbance against deionized water.

**Step III - Total Hemoglobin (THb) assay**

1. Pipette 5.0 ml of deionized water into a test tube.
2. Add to it 0.02 ml of hemolysate (from step 1).
3. Mix and read absorbance against deionized water.

**Calculation:**

\[
GHb\% = \frac{A \text{ of GHb}}{A \text{ of THb}} \times 10 \times \text{Temperature factor (Tf)}
\]

For assay at 23°C Tf = 1.0; at 30°C Tf = 0.9

v. **Measurement of Insulin resistance index:** Insulin resistance was calculated by HOMA index for measurement of insulin sensitivity on 7th, 10th, 14th, 16th, 18th, 20th, 22nd and 24th week (Luo et al., 2011).

\[
HOMA = \frac{\text{Fasting insulin (mU/l)}}{22.5} \times \text{Fasting glucose (mmol/L)}
\]

vi. **Assessment of lipid profile**

Lipid profile (Cholesterol, HDL, LDL and triglycerides) was measured by using diagnostic kits (ERBA Diagnostics, Mannheim GmbH, Germany) on 7th, 10th, 14th, 16th, 18th, 20th, 22nd and 24th week.

**Cholesterol**

Clinical significance of measurement of serum cholesterol levels can serve as an indicator of liver function, biliary function, intestinal absorption, propensity towards coronary artery disease, thyroid function and adrenal disease. Cholesterol levels are important in the diagnosis and classification of hyperlipoproteinaemias. Stress, age, gender, hormonal balance and pregnancy affect normal cholesterol levels.

**Principle**

This reagent is based on the formulation of Allain et al. (1974) and the modification of Roeschlaub et al. (1974) with further improvements to render the reagent stable in solution.

1. Cholesterol esters are enzymatically hydrolysed by cholesterol esterase to cholesterol and free fatty acids.
2. Free cholesterol, including that originally present, then oxidized by cholesterol oxidase to cholest-4-en-3-one and hydrogen peroxide.

3. The hydrogen peroxide combines with 4-aminoantipyrine to form a chromophore (quinoneimine dye) which may be quantitated at 505 nm.

Cholesterol ester + H₂O → Cholesterol Esterase → Cholesterol + Fatty acids

Cholesterol + O₂ → Cholesterol Oxidase → Cholest-4-en-3-one + H₂O₂

2H₂O₂ + 4AAP + Phenol → Peroxidase → Quinoneimine dye + 4H₂O

Reagent Composition

R1
- Good’s Buffer: 50 mmol/l
- Phenol: 5 mmol/l
- 4-aminoantipyrine: 0.3 mmol/l
- Cholesterol esterase: ≥ 200 U/l
- Cholesterol oxidase: ≥ 50 U/l
- Peroxidase: ≥ 3 kU/l

Assay procedure

Wavelength: 500 (546) nm

<table>
<thead>
<tr>
<th></th>
<th>Reagent blank</th>
<th>Standard (Calibrator)</th>
<th>Sample</th>
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<tbody>
<tr>
<td>Reagent 1</td>
<td>1.00 ml</td>
<td>1.00 ml</td>
<td>1.00 ml</td>
</tr>
<tr>
<td>Sample</td>
<td>-</td>
<td>-</td>
<td>0.01 ml</td>
</tr>
<tr>
<td>Standard (Calibrator)</td>
<td>-</td>
<td>0.01 ml</td>
<td>-</td>
</tr>
<tr>
<td>Distilled water</td>
<td>0.01 ml</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Mix and incubate 10 min. at 37°C. Measure absorbance of the sample and standard against reagent blank. The coloration is stable during one hour.
**Calculation**

\[
\text{Cholesterol (mg/dl)} = \frac{\Delta A_{\text{sample}}}{\Delta A_{\text{standard}}} \times \text{Standard (calibrator) concentration}
\]

\(\Delta A = \text{Difference in absorbance}\)

Measure absorbance of the sample and standard against reagent blank. The coloration is stable during one hour.

**HDL direct**

**Clinical significance**

High-density lipoproteins (HDL) compose one of the major classes of plasma lipoproteins. They are synthesized in liver as complexes of apolipoprotein and phospholipid and are capable of picking up cholesterol and carrying it from arteries to the liver, where the cholesterol is converted to bile acids and excreted into the intestine.

**Principle**

The assay is based on a modified polyvinyl sulfonic acid (PVS) and polyethylene-glycol-methyl ether (PEGME) coupled classic precipitation method with the improvements in using optimized quantities of PVS/PEGME and selected detergents. LDL, VLDL and chylomicron (CM) react with PVS and PEGME and the reaction results in inaccessibility of LDL, VLDL and CM by cholesterol oxidase (CHOD) and cholesterol esterase (CHER). The enzymes selectively react with HDL to produce \(\text{H}_2\text{O}_2\) which is detected through a Trinder reaction.

\[
\begin{align*}
\text{HDL + LDL + VLDL + CM} & \xrightarrow{\text{PVS, PEGME}} \text{HDL + (LDL + VLDL + CM) + PVS, PEGME} \\
\text{HDL} & \xrightarrow{\text{CHOD, CHER}} \text{Fatty Acid + H}_2\text{O}_2 \\
2\text{H}_2\text{O}_2 + 4\text{-AA} + \text{TODB} & \xrightarrow{\text{Peroxidase}} \text{Quinone + 5 H}_2\text{O}
\end{align*}
\]

**Assay procedure**
Chapter 4A

<table>
<thead>
<tr>
<th>Pipette in Tube</th>
<th>Reagent blank</th>
<th>Sample / Calibrator</th>
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<tbody>
<tr>
<td>Reagent 1</td>
<td>750 μl</td>
<td>750 μl</td>
</tr>
<tr>
<td>D.D water</td>
<td>10 μl</td>
<td>-</td>
</tr>
<tr>
<td>Sample / Calibrator</td>
<td>-</td>
<td>10 μl</td>
</tr>
</tbody>
</table>

Mix and incubate at 37°C for 5 min.

Add Reagent 2  250 μl  250 μl

Mix and incubate at 37°C for 5 min.

Read final absorbances at the specified wavelength against reagent blank.

**Calculation**

\[
\text{HDL} - C = \frac{\text{Abs. of Sample} - \text{Abs. of Sample Blank}}{\text{Abs. of Cal.} - \text{Abs. of Cal. Blank}} \times \text{Concentration of Calibrator}
\]

(HDL-C = HDL-Cholesterol)

**LDL**

**Clinical significance**

Low Density Lipoproteins (LDL) are synthesized in the liver by the action of various lipolytic enzymes on triglyceride-rich Very Low Density Lipoproteins (VLDLs). Specific LDL receptors exist to facilitate the elimination of LDL from plasma by liver parenchymal cells. It has been shown that most of the cholesterol stored in atherosclerotic plaques originates from LDL. For this reason the LDL Cholesterol concentration is considered to be the most important clinical predictor, of all single parameters, with respect to coronary atherosclerosis.

Accurate measurement of LDL Cholesterol is of vital importance in therapies which focus on lipid reduction to prevent atherosclerosis or reduce its progress and to avoid plaque rupture.

**Principle**

The assay is based on a modified polyvinyl sulfonic acid (PVS) and polyethylene-glycol methyl ether (PEGME) coupled classic precipitation method with the improvements in using optimized quantities of PVS/PEGME
and selected detergents. LDL, VLDL, and chylomicron (CM) react with PVS and PEGME and the reaction results in inaccessibility of LDL, VLDL and CM by cholesterol oxidase (CHOD) and cholesterol esterase (CHER), whereas HDL reacts with the enzymes. Addition of R2 containing a specific detergent releases LDL from the PVS/PEGME complex. The released LDL reacts with the enzymes to produce H2O2 which is quantified by the Trinder reaction.

\[
\text{HDL} + \text{LDL} + \text{VLDL} + \text{CM} \xrightarrow{\text{PVS, PEGME}} \text{HDL} + (\text{LDL} + \text{VLDL} + \text{CM}) \cdot \text{PVS/PEGME}
\]

\[
\text{CHOD, CHER}
\]

\[
\text{HDL} \quad \text{Fatty Acid} + \text{H}_2\text{O}_2
\]

\[
\text{Peroxidase}
\]

\[
\text{2H}_2\text{O}_2 + 4\text{-AA} + \text{TODB} \xrightarrow{\text{Peroxidase}} \text{Quinone} + 5\text{H}_2\text{O}
\]

**Reagent composition**

**R1**
- MES buffer (pH 6.5) 50 mmol/l
- Polyvinylsulfonic acid 50 mg/l
- Polyethyleneglycolmethylester 30 ml/l
- 4-aminoantipyrine 0.9 g/l
- Cholesterol esterase 5 kU/l
- Cholesterol oxidase 20 kU/l
- Peroxidase 5 kU/l
- Detergent

**R2**
- MES buffer (pH 6.5) 50 mmol/l
- Detergent
- TODB N,N-Bis(4-sulfobutyl)-3-methylaniline 3 mmol/l

**ASSAY PROCEDURE**
### Chapter 4A

<table>
<thead>
<tr>
<th>Pipette in Tube</th>
<th>Reagent blank</th>
<th>Sample / Calibrator</th>
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</thead>
<tbody>
<tr>
<td>Reagent 1</td>
<td>750 µl</td>
<td>750 µl</td>
</tr>
<tr>
<td>D.D water</td>
<td>6 µl</td>
<td>-</td>
</tr>
<tr>
<td>Sample / Calibrator</td>
<td>-</td>
<td>6 µl</td>
</tr>
</tbody>
</table>

Mix and incubate at 37°C for 5 min.

Add Reagent 2

<table>
<thead>
<tr>
<th></th>
<th>250 µl</th>
<th>250 µl</th>
</tr>
</thead>
</table>

Mix and incubate at 37°C for 5 min.

Read final absorbances at the specified wavelength against reagent blank.

**Calculation**

\[
\text{LDL} - C = \frac{\text{Abs. of Sample} - \text{Abs. of Sample Blank}}{\text{Abs. of Cal.} - \text{Abs. of Cal. Blank}} \times \text{Concentration of Calibrator}
\]

**Triglycerides estimation:**

**Clinical Significance**

Triglycerides are a family of lipids absorbed from the diet and produced endogenously from carbohydrates. Measurement of triglycerides is important in the diagnosis and management of hyperlipidemias. These diseases can be genetic or secondary to other disorders including nephrosis, diabetes mellitus and endocrine disturbances. Elevation of triglycerides has been identified as a risk factor for atherosclerotic disease.

**Principle**

The series of reactions involved in the assay system is as follows:

\[
\text{Triglycerides + H}_2\text{O} \xrightarrow{\text{LPL}} \text{Glycerol + Free Fatty acids}
\]

\[
\text{Glycerol + ATP} \xrightarrow{\text{GK}} \text{Glycerol-3-phosphate + ADP}
\]
Triglycerides are enzymatically hydrolyzed by lipase to free acids and glycerol. The glycerol is phosphorylated by adenosine triphosphate (ATP) with glycerol kinase (GK) to produce glycerol-3-phosphate and adenosine diphosphate (ADP). Glycerol-3-phosphate is oxidized to dihydroxy-acetone phosphate (ADP) by glycerol phosphate oxidase producing hydrogen peroxide (H$_2$O$_2$).

In a Trinder type colour reaction catalyzed by peroxidase, the H$_2$O$_2$ reacts with 4-aminoantipyrine (4AAP) and 4-chlorophenol to produce a red coloured dye. The absorbance of this dye is proportional to the concentration present in the sample.

**Unit Conversion**

\[
\text{mg/dl} \times 0.0113 = \text{mmol/l}
\]

HbA1c level was measured by cation exchange resin method (Excel Diagnostics, Hyderabad) on 7th, 10th, 14th, 16th, 18th, 20th, 22nd and 24th weeks and the experiments were conducted following the manufacturer’s instructions.

**4.1.2.4.  Assessment of behavioral paradigms:**

Memory impairment was assessed in two behavioural paradigms (Morris water maze and elevated plus maze):

**4.1.2.4.1. Morris water maze (computer tracking using EthoVision software)**

Animals were tested in a spatial version of Morris water maze test for assessment of memory (Morris et al., 1982; Tuzcu and Baydas, 2006) on 7th, 14th, 16th, 18th, 20th, 22nd and 24th weeks. For detail refer Chapter 1 (1.2.5.1.)
4.1.2.4.2. **Memory consolidation test**

Refer Chapter 1 (1.2.5.2.)

4.1.2.4.3. **Elevated plus maze test**

Memory acquisition and retention were tested using elevated plus maze test on 7th, 14th, 16th, 18th, 20th, 22nd and 24th weeks. Refer Chapter 1 (1.2.5.3.)

4.1.2.5. **Biochemical assessment**

4.1.2.5.1. **Acetylcholinesterase activity**

Refer to Chapter 1 (1.2.6.1.).

4.1.2.5.2. **Assessment of oxidative stress**

Refer to Chapter 1 (1.2.6.2.)

4.1.2.5.3. **Assessment of nitrosative stress**

Refer to Chapter 1 (1.2.6.3.).

4.1.2.5.4. **Protein estimation**

Refer to Chapter 1 (1.2.6.4.)

4.1.2.6. **Molecular estimation by ELISA kits**

4.1.2.6.1. **Estimation of Tumor Necrosis Factor-alpha (TNF-α)**

The quantification of TNF-α in rat serum was done on 7th, 10th, 14th, 16th, 18th, 20th, 22nd and 24th weeks and hippocampal TNF-α on 24th week, according to manufacturer's instructions provided with Quantikine Rat TNF-α immunoassay kits (R&D Systems, USA). Refer to Chapter 1 (1.2.7.1).

4.1.2.6.2. **Estimation of Transforming Growth Factor beta 1 (TGF-β1)**

The quantification of TGF-β1 in rat serum was done on 7th, 10th, 14th, 16th, 18th, 20th, 22nd and 24th weeks and hippocampal TGF-β1 levels on 24th week, according to manufacturer's instructions provided with Quantikine Rat TGF-β1 immunoassay kits (R&D Systems, USA). Refer to Chapter 1 (1.2.7.2).
4.1.2.6.3. Estimation of Interleukin-1beta (IL-1β)

The quantification of IL-1β in rat serum was done on 7th, 10th, 14th, 16th, 18th, 20th, 22nd and 24th weeks and hippocampal IL-1β level on 24th week, according to manufacturer's instructions provided with Quantikine Rat IL-1β immunoassay kits (R&D Systems, USA). Refer to Chapter 1 (1.2.7.3).

4.1.2.6.4. Quantification of NF-κβ p65 unit

The quantification of NF-κβ p65 unit were measured in rat hippocampal and cortical region on 22nd and 24th week, according to manufacturer's instructions provided with NF-κβ/p65 ActivELISA (Imgenex, San Diego, USA) kit. Refer to Chapter 1 (1.2.7.4).

4.1.2.6.5. Caspase-3 Colorimetric Assay

Caspase-3 activity was measured in rat hippocampal and cortical region on 22nd and 24th week, by using assay kit of Biovision, USA, according to manufacturer's instructions. Refer to Chapter 1 (1.2.7.5).

4.1.2.6.6. Serum homocysteine (Hcy)

Serum Hcy levels were measured on 7th, 10th, 14th, 16th, 18th, 20th, 22nd and 24th weeks using Rat Hcy ELISA kit (DRG, Germany). The analysis was done according to the manufacturer's instructions. The results were expressed as pmol/l.

4.1.2.7. Statistical analysis

Results were expressed as mean ± S.E.M. The intergroup variation was measured by one-way analysis of variance (ANOVA) followed by Tukey's test using GraphPad Prism® statistical software version 5.01. Two-way ANOVA followed by Tukey's test was employed to discover the inter-group variation in escape latency and path length of the Morris water maze by GraphPad Prism® statistical software version 5.01. A value of p<0.05 was considered statistically significant.
4.1.3. RESULTS

4.1.3.1. Effect of fructose on basic physiological parameters:

Fructose fed animals exhibited increased plasma glucose, insulin and glycosylated hemoglobin levels in time dependent manner. Insulin resistance was calculated by HOMA-index and significant elevation was observed at 7th week. Further, it was evidenced by marked increase in body weight as well as altered lipid profile in a time dependent manner. Systolic blood pressure was significantly increased at 24 weeks in fructose drinking rats (164.85 mmHg ± 3.19) as compared to control (117.83 mmHg ± 3.97). These observations clearly demonstrated marked alterations in physiological parameters due to chronic fructose consumption (Table 4.1.1).
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Glucose (mg/dl)</th>
<th>Insulin (pmol/l)</th>
<th>HbA1c (%)</th>
<th>B. Wt (g)</th>
<th>Body weight, [F(13, 70) = 874.06 (p &lt; 0.001)]</th>
<th>Water intake (ml)</th>
<th>Food intake (g)</th>
<th>Triglycerides (mg/dl)</th>
<th>HDL (mg/dl)</th>
<th>LDL (mg/dl)</th>
<th>Cholesterol (mg/dl)</th>
<th>HOMA-IR Index</th>
<th>HOMA-β Index</th>
</tr>
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<tr>
<td>Control-1</td>
<td>7.2 ± 0.37</td>
<td>25 ± 0.71</td>
<td>25 ± 0.51</td>
<td>97.6 ± 1.1</td>
<td></td>
<td>256.8 ± 4.56</td>
<td>92 ± 3.2</td>
<td>130 ± 0.1</td>
<td>98.9 ± 2.7</td>
<td>51 ± 1.96</td>
<td>238 ± 4.49</td>
<td>4.4 ± 0.14</td>
<td>181.06 ± 4.01</td>
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<td>181.06 ± 4.01</td>
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<td>Control-10</td>
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<td>256.8 ± 4.56</td>
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<td>Fructose-10</td>
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<td>130 ± 0.1</td>
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<td>256.8 ± 4.56</td>
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<td>130 ± 0.1</td>
<td>98.9 ± 2.7</td>
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<td>256.8 ± 4.56</td>
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<td>130 ± 0.1</td>
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<td>51 ± 1.96</td>
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<tr>
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<td>256.8 ± 4.56</td>
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<td>130 ± 0.1</td>
<td>98.9 ± 2.7</td>
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<tr>
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<td>92 ± 3.2</td>
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<td>98.9 ± 2.7</td>
<td>51 ± 1.96</td>
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<tr>
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<td>256.8 ± 4.56</td>
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<td>51 ± 1.96</td>
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<td>Fructose-18</td>
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<tr>
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<tr>
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<td>256.8 ± 4.56</td>
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<td>98.9 ± 2.7</td>
<td>51 ± 1.96</td>
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<td>98.9 ± 2.7</td>
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<tr>
<td>Control-24</td>
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<td>256.8 ± 4.56</td>
<td>92 ± 3.2</td>
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<td>98.9 ± 2.7</td>
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<tr>
<td>Fructose-24</td>
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<td>25 ± 0.51</td>
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<td>256.8 ± 4.56</td>
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<td>130 ± 0.1</td>
<td>98.9 ± 2.7</td>
<td>51 ± 1.96</td>
<td>238 ± 4.49</td>
<td>4.4 ± 0.14</td>
<td>181.06 ± 4.01</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM. Body weight, [F(13, 70) = 874.06 (p < 0.001)]; water intake, [F(13, 70) = 718.32 (p < 0.001)]; insulin, [F(13, 70) = 168.06 (p < 0.001)]; HbA1c, [F(13, 70) = 168.06 (p < 0.001)]; HOMA-IR, [F(13, 70) = 168.06 (p < 0.001)]; total cholesterol, [F(13, 70) = 168.06 (p < 0.001)]. a represents difference from respective control group (p < 0.05), b represents difference from fructose 7 week (p < 0.05), c represents difference from fructose 10 week (p < 0.05), d represents difference from fructose 14 week (p < 0.05), e represents difference from fructose 16 week (p < 0.05), f represents difference from fructose 20 week (p < 0.05), g represents difference from fructose 22 week (p < 0.05), h represents difference from fructose 24 week (p < 0.05).
4.1.3.2. Behavioral observations

4.1.3.2.1. Effect of fructose on performance in Morris water maze task

The cognitive function was assessed in the Morris water maze test. The mean escape latency did not differ between any of the groups on the first day of testing in Morris water maze but from second day onwards, although no significant difference in transfer latency was observed in 7th, 14th, 16th and 18th week group but significant difference in transfer latency was observed after 20th, 22nd and 24th weeks. Rats showed a poorer learning performance to find out the platform in the 5th day of training after 20th week of fructose (15% w/v) administration which was continued till 24th week \[F(13, 70) = 46.82 \ (p < 0.001)\] (Fig. 4.1.1).

![Figure 4.1.1](image_url) Effect of fructose feeding at 7th, 14th, 16th, 18th, 20th, 22nd, 24th week on escape latency in Morris water maze on day 5. Values were expressed as mean ± SEM. 7th = 7th week; 14th = 14th week; 16th = 16th week; 18th = 18th week; 20th = 20th week; 22nd = 22nd week; 24th = 24th week. a represents difference from respective control group (p < 0.05), b represents difference from fructose 20th week, c represents difference from fructose 22nd week.
4.1.3.2.2. *Effect of fructose on total distance travelled to reach the hidden platform (path length)*

Progressive decrease in path length to reach the hidden platform on subsequent days in water maze task is associated with intact memory of animals. The total distance travelled to reach the hidden platform did not differ between any of the time point on the first day of testing in Morris water maze but from second day onwards, there was significant difference in path length at 20\textsuperscript{th}, 22\textsuperscript{nd} and 24\textsuperscript{th} week of fructose feeding as compared to control group (Fig.4.1.2). However, no significant difference was observed in path length in probe trial between control and fructose group at 7\textsuperscript{th}, 14\textsuperscript{th}, 16\textsuperscript{th}, 18\textsuperscript{th} weeks. \[F(13, 70) = 126.04 \ (p < 0.001)\].

![Figure 4.1.2](image.png)

**Figure 4.1.2** Effect of fructose feeding at 7\textsuperscript{th}, 14\textsuperscript{th}, 16\textsuperscript{th}, 18\textsuperscript{th}, 20\textsuperscript{th}, 22\textsuperscript{nd}, 24\textsuperscript{th} week on path length in Morris water maze on day 5. Values were expressed as mean ± SEM. 7\textsuperscript{th} = 7\textsuperscript{th} week; 14\textsuperscript{th}=14\textsuperscript{th} week; 16\textsuperscript{th}=16\textsuperscript{th} week; 18\textsuperscript{th}= 18\textsuperscript{th} week; 20\textsuperscript{th}= 20\textsuperscript{th} week; 22\textsuperscript{nd}= 22\textsuperscript{nd} week; 24\textsuperscript{th}= 24\textsuperscript{th} week. a represents difference from respective control group (p < 0.05), b represents difference from fructose 20\textsuperscript{th} week, c represents difference from fructose 22\textsuperscript{nd} week.
<table>
<thead>
<tr>
<th>Weeks</th>
<th>Control</th>
<th>FR</th>
</tr>
</thead>
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<tr>
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</tr>
<tr>
<td>18th</td>
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</tr>
<tr>
<td>24th</td>
<td><img src="image13" alt="Diagram" /></td>
<td><img src="image14" alt="Diagram" /></td>
</tr>
</tbody>
</table>

**Figure 4.1.3.** Effect of fructose feeding at 7th, 14th, 16th, 18th, 20th, 22nd, 24th week on the swimming pattern on day 5th in morris water maze. 7th = 7th week; 14th = 14th week; 16th = 16th week; 18th = 18th week; 20th = 20th week; 22nd = 22nd week; 24th = 24th week.
4.1.3.2.3. Effect of fructose on time spent in target quadrant

In the probe trial also, which measures how well the animals had learned and consolidated the platform location during the training, animals showed no difference after 7th, 14th, 16th and 18th weeks of fructose feeding but after 20th, 22nd and 24th week, significant difference was observed. The time spent in the target quadrant was significantly lower after 20 weeks in fructose fed rats as compared to the control group \[F(13, 70) = 43.88 (p < 0.001)\] (Fig 4.1.4).

![Figure 4.1.4](image)

**Figure 4.1.4** Effect of fructose feeding at 7th, 14th, 16th, 18th, 20th, 22nd, 24th week on time spent in target quadrant in Morris water maze. Values were expressed as mean ± SEM. 7th = 7th week; 14th = 14th week; 16th = 16th week; 18th = 18th week; 20th = 20th week; 22nd = 22nd week; 24th = 24th week. a represents difference from respective control group (p < 0.05), b represents difference from fructose 20th week.
4.1.3.2.4. Effect of fructose on memory assessment in elevated plus maze test

Percent initial transfer latency did not differ significantly after 7th, 14th, 16th and 18th weeks of fructose administration. However, a significant difference was observed after 20th, 22nd and 24th weeks of fructose consumption \[F(13, 70) = 12.89 \text{ (p <0.001)}\] (Fig. 4.1.5).

![Graph showing effect of fructose on percent initial transfer latency in elevated plus maze test.](image)

**Figure 4.1.5** Effect of fructose feeding at 7th, 14th, 16th, 18th, 20th, 22nd, 24th week on percent initial transfer latency in elevated plus maze. Values were expressed as mean ± SEM. Values were expressed as mean ± SEM. 7th = 7th week; 14th = 14th week; 16th = 16th week; 18th = 18th week; 20th = 20th week; 22nd = 22nd week; 24th = 24th week. a represents difference from respective control group (p < 0.05).

4.1.3.2.5. Effect of fructose on the locomotor activity

The spontaneous locomotor activity did not differ significantly between the control and fructose administration for 24 weeks \[F(13, 70) = 0.672 \text{ (p =0.67)}\] (Fig.4.1.6).
4.1.3.3. **Biochemical and molecular observations**

4.1.3.3.1. **Effect of fructose on acetylcholinesterase activity**

Acetylcholinesterase activity was significantly elevated in cerebral cortex and hippocampus of fructose fed rats as compared to control group (Table 4.1.2) There was a statistically significant difference among the mean value of control and fructose fed group in cerebral cortex \[F(3, 20) = 5.965(p <0.001)\] and hippocampus \[F(2,14) = 6.588 (p <0.001)].

4.1.3.3.2. **Effect of fructose on brain lipid peroxidation**

Thiobarbituric acid reactive substance levels were increased significantly in cerebral cortex and hippocampus of fructose fed rats as compared to control group (Table 4.1.2). There was a statistically significant
difference among the mean value of control and fructose group. cerebral cortex \[F(2,14) = 16819.82 \ (p <0.001)\] and hippocampus \[F(2,14) = 13957.26 \ (p <0.001)\].

4.1.3.3.3. **Effect of fructose on nitrosative stress in rat brains**

Total nitric oxide level was significantly elevated in cerebral cortex and hippocampus of fructose fed rats as compared to control group (Table 4.1.2). There was a statistically significant difference among the mean value of control and fructose exposed group in cerebral cortex \[F(2,14) = 195.28 \ (p <0.001)\] and hippocampus \[F(2,14) = 204.23 \ (p <0.001)\]. However, not a statistically significant difference was found between hippocampal and cortical nitrite levels between 22 and 24 weeks of fructose rats.

4.1.3.3.4. **Effect of fructose on antioxidant profile of rat brains**

The non protein thiols and enzyme activity of superoxide dismutase and catalase was significantly decreased in cerebral cortex and hippocampus of fructose fed rats as compared to control group. Non protein thiols, cortex \[F(3, 20) = 488.48 \ (p <0.001)\]; hippocampus \[F(3, 20) = 109.28 \ (p <0.001)\], superoxide dismutase cortex\[F(3, 120) = 147.37 \ (p <0.001)\]; hippocampus \[F(2,14) = 130.27 \ (p <0.001)\]; catalase, cortex \[F(3, 20) = 56.52 \ (p <0.001)\]; hippocampus \[F(3, 20) = 113.87 \ (p <0.001)\] (Table 4.1.2).
Table 4.1.2: Effect of fructose administration on lipid peroxides, glutathione, superoxide dismutase, catalase, nitrite and acetylcholinesterase (AChE) activity, in cerebral cortex and hippocampus. Values were expressed as mean ± SEM. Control 22 = control 22 week; Control 24 = control 24 week; FR-22= Fructose 22 week; FR-24= Fructose 24 week; a represents difference from respective control p<0.05.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Brain</th>
<th>LPO (nmoles/mg protein)</th>
<th>GSH (µmoles/mg protein)</th>
<th>SOD (Units/mg protein)</th>
<th>Catalase (µM of H₂O₂/min/mg protein)</th>
<th>Brain Nitrite (µg/ml)</th>
<th>AChE (µM/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hippo</td>
<td>1.79 ± 0.014</td>
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<td>5.50 ± 0.37</td>
<td>11.99 ± 0.74</td>
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<td>Cortex</td>
<td>1.65 ± 0.02</td>
<td>0.283 ± 0.02</td>
<td>5.69 ± 0.36</td>
<td>12.65 ± 0.57</td>
<td>185.43 ± 12.42</td>
<td>0.014 ± 0.012</td>
</tr>
<tr>
<td></td>
<td>Hippo</td>
<td>6.25 ± 0.03³</td>
<td>0.66 ± 0.01³</td>
<td>0.64 ± 0.17³</td>
<td>2.42 ± 0.72³</td>
<td>853.56 ± 31.27³</td>
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<tr>
<td>FR-22</td>
<td>Cortex</td>
<td>5.66 ± 0.022³</td>
<td>0.06 ± 0.012²</td>
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<td>Hippo</td>
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<td>2.52 ± 0.55³</td>
<td>899.33 ± 34.73³</td>
<td>0.03 ± 0.001³</td>
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4.1.3.3.5. Effect of fructose on serum TNF-α levels:

Rats subjected to fructose feeding showed a significant and time dependent increase in serum TNF-α [$F(13,70) = 348.50 \ (p < 0.001)$] level as compared with control group after $7^{th}$, $10^{th}$, $14^{th}$, $16^{th}$, $18^{th}$, $20^{th}$, $22^{nd}$ and $24^{th}$ weeks of fructose drinking (Fig. 4.1.7).

![Figure 4.1.7: Effect of fructose administration on serum TNF-α level. Values were expressed as mean ± SEM. $1^{st}$ = 1$^{st}$ week; $7^{th}$ = 7$^{th}$ week; $10^{th}$ = 10$^{th}$ week; $14^{th}$ = 14$^{th}$ week; $16^{th}$ = 16$^{th}$ week; $18^{th}$ = 18$^{th}$ week; $20^{th}$ = 20$^{th}$ week; $22^{nd}$ = 22$^{nd}$ week; $24^{th}$ = 24$^{th}$ week. a represents difference from respective control group (p < 0.05), b represents difference from fructose 7$^{th}$ week (p < 0.05), c represents difference from fructose 10$^{th}$ week (p < 0.05), d represents difference from fructose 14$^{th}$ week (p < 0.05), e represents difference from fructose 16$^{th}$ week (p < 0.05), f represents difference from fructose 18$^{th}$ week (p < 0.05), g represents difference from fructose 20$^{th}$ week (p < 0.05), h represents difference from fructose 22$^{nd}$ week (p < 0.05).]
Figure 4.1.8: Effect of fructose administration on serum TGF-β level. Values were expressed as mean ± SEM. 1<sup>st</sup> = 1<sup>st</sup> week; 7<sup>th</sup> = 7<sup>th</sup> week; 10<sup>th</sup> = 10<sup>th</sup> week; 14<sup>th</sup> = 14<sup>th</sup> week; 16<sup>th</sup> = 16<sup>th</sup> week; 18<sup>th</sup> = 18<sup>th</sup> week; 20<sup>th</sup> = 20<sup>th</sup> week; 22<sup>nd</sup> = 22<sup>nd</sup> week; 24<sup>th</sup> = 24<sup>th</sup> week. a represents difference from respective control group (p < 0.05), b represents difference from fructose 7<sup>th</sup> week (p < 0.05), c represents difference from fructose 10<sup>th</sup> week (p < 0.05), d represents difference from fructose 14<sup>th</sup> week (p < 0.05), e represents difference from fructose 16<sup>th</sup> week (p < 0.05), f represents difference from fructose 18<sup>th</sup> week (p < 0.05), g represents difference from fructose 20<sup>th</sup> week (p < 0.05), h represents difference from fructose 22<sup>nd</sup> week (p < 0.05).

4.1.3.3.7. Effect of fructose on IL-1β levels:

Rats subjected to fructose feeding showed a significant and time dependent increase in IL-1β [F(13,70) = 113.08 (p < 0.001)] level as compared with control group after 7<sup>th</sup>, 10<sup>th</sup>, 14<sup>th</sup>, 16<sup>th</sup>, 18<sup>th</sup>, 20<sup>th</sup>, 22<sup>nd</sup> and 24<sup>th</sup> weeks of fructose drinking (Fig. 4.1.9).
Figure 4.1.9: Effect of fructose administration on serum IL-1β level. Values were expressed as mean ± SEM. 1\textsuperscript{st} = 1\textsuperscript{st} week; 7\textsuperscript{th} = 7\textsuperscript{th} week; 10\textsuperscript{th} = 10\textsuperscript{th} week; 14\textsuperscript{th}=14\textsuperscript{th} week; 16\textsuperscript{th}=16\textsuperscript{th} week; 18\textsuperscript{th}=18\textsuperscript{th} week; 20\textsuperscript{th}=20\textsuperscript{th} week; 22\textsuperscript{nd}=22\textsuperscript{nd} week; 24\textsuperscript{th}=24\textsuperscript{th} week. a represents difference from respective control group (p < 0.05), b represents difference from fructose 7\textsuperscript{th} week (p < 0.05), c represents difference from fructose 10\textsuperscript{th} week (p < 0.05), d represents difference from fructose 14\textsuperscript{th} week (p < 0.05), e represents difference from fructose 16\textsuperscript{th} week (p < 0.05), f represents difference from fructose 18\textsuperscript{th} week (p < 0.05), g represents difference from fructose 20\textsuperscript{th} week (p < 0.05), h represents difference from fructose 22\textsuperscript{nd} week (p < 0.05).

4.1.3.3.8. Effect of fructose on homocysteine levels:

Rats subjected to fructose feeding showed a significant and time dependent increase in serum homocysteine \[F(13,70) = 145.22 \text{ (p < 0.0001)}\] level as compared with control group after 7\textsuperscript{th}, 10\textsuperscript{th}, 14\textsuperscript{th}, 16\textsuperscript{th}, 18\textsuperscript{th}, 20\textsuperscript{th}, 22\textsuperscript{nd} and 24\textsuperscript{th} weeks of fructose drinking (Fig. 4.1.10).
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Figure 4.1.10: Effect of fructose administration on serum homocysteine level. Values were expressed as mean ± SEM. 1\textsuperscript{st} = 1\textsuperscript{st} week; 7\textsuperscript{th} = 7\textsuperscript{th} week; 10\textsuperscript{th} = 10\textsuperscript{th} week; 14\textsuperscript{th} = 14\textsuperscript{th} week; 16\textsuperscript{th} = 16\textsuperscript{th} week; 18\textsuperscript{th} = 18\textsuperscript{th} week; 20\textsuperscript{th} = 20\textsuperscript{th} week; 22\textsuperscript{nd} = 22\textsuperscript{nd} week; 24\textsuperscript{th} = 24\textsuperscript{th} week. a represents difference from respective control group (p < 0.05), d represents difference from fructose 14\textsuperscript{th} week (p < 0.05), e represents difference from fructose 16\textsuperscript{th} week (p < 0.05), f represents difference from fructose 18\textsuperscript{th} week (p < 0.05), g represents difference from fructose 20\textsuperscript{th} week (p < 0.05), h represents difference from fructose 22\textsuperscript{nd} week (p < 0.05).

4.1.3.3.9. Effect of 24 weeks fructose feeding on hippocampal pro-inflamatory cytokines

Rats subjected to fructose feeding for 24 weeks showed a significant increase on hippocampal pro-inflammatory cytokines (Table 4.1.3).

Table 4.1.3: Effect of 24 weeks fructose feeding on hippocampal pro-inflammatory cytokines. a represents difference from control p<0.05.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Hippocampal TNF-α</th>
<th>Hippocampal TGF-β</th>
<th>Hippocampal IL-1β</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>75.3 ± 3.52</td>
<td>205.7 ± 8.97</td>
<td>22.1 ± 3.52</td>
</tr>
<tr>
<td>Fructose 24 weeks</td>
<td>335.4 ± 4.57\textsuperscript{a}</td>
<td>662.4 ± 7.18\textsuperscript{a}</td>
<td>79.2 ± 5.11\textsuperscript{a}</td>
</tr>
</tbody>
</table>
4.1.3.3.10. Effect of fructose consumption on nuclear factor kappa beta (NFκβ) and caspase-3 activity

NF-κβ p65 subunit expression was increased 2.9 and 2.4 fold in hippocampus and cerebral cortex respectively after 24 weeks in fructose fed rats. In addition, caspase-3 activity was 3.5 fold increased in hippocampus and 3.7 fold increased in cerebral cortex after 24 weeks of fructose administration (Fig 4.1.11).

Figure 4.1.11: Effect of fructose drinking on NFκβ (A) and caspase-3 activity (B) Values were expressed as mean ± SEM, a represents, difference from control p<0.05.
4.1.3.3.11. Correlation between elevated triglycerides levels and cognitive function

Pearson correlation revealed that serum TG concentrations were positively correlated with retention deficits at 20th, 22nd and 24th weeks of fructose feeding. This indicates a triglyceride association with memory (Fig. 4.1.12).

![Graph showing correlation between escape latency and triglycerides](image)

**Figure 4.1.12:** Pearson correlation between escape latency vs triglycerides. FR-7 = Fructose 7th week; FR-14 = Fructose 14th week; FR-16 = Fructose 16th week; FR-18 = Fructose 18th week; FR-20 = Fructose 20th week; FR-22 = Fructose 22nd week; FR-24 = Fructose 24th week.

4.1.3.3.12. Association between fructose-induced insulin resistance and cognitive function

Insulin resistance index was found to be positively correlated with latencies to reach the target especially at 18th, 20th 22nd and 24th weeks of fructose feeding, implying that cognitive function relies on the levels of insulin resistance index (Fig.4.1.13).
Figure 4.1.13: Pearson correlation between escape latency vs insulin resistance. 7th week; FR-14= Fructose 14th week; FR-16= Fructose 16th week; FR-18= Fructose 18th week; FR-20= Fructose 20th week; FR-22= Fructose 22nd week; FR-24= Fructose 24th week.
4.1.4. DISCUSSION

It is being increasingly recognized that aberrant body metabolism can disrupt brain plasticity and cognition (Agrawal and Gomez-Pinilla, 2012). The present study for the first time demonstrates a relationship between duration of insulin resistance and development of associated cognitive dysfunction in male rats fed with 15% fructose for a period of 24 weeks. We found that, 15% fructose solution as a substitute for drinking water time dependently and progressively increased plasma glucose and insulin level coupled with a graded increase in HOMA-IR index which was measured at 1st, 7th, 10th, 14th, 16th, 18th, 20th, 22nd and 24th weeks during the study.

Hwang and colleagues (1987) first reported that rats fed a high fructose diet exhibited insulin resistance, hyperinsulinemia, hypertriglyceridemia and hypertension (Hwang et al., 1987; Tran et al., 2009). In our study, parameters of insulin resistance i.e. body weight, plasma insulin, blood glucose, glycosylated heamoglobin, blood pressure, deranged lipid profile and HOMA-IR index became significantly elevated at 7th week of fructose feeding and kept on increasing till 24 weeks. However, the escape latency and total distance travelled (swim path) to reach the hidden platform in Morris water maze task remained unaltered at 14th, 16th and 18th week but significantly increased on 20th, 22nd and 24th week. This indicates that although insulin resistance became evident on 7th week of fructose feeding but decline in spatial memory appeared on 20th week. These observations reveal that sufficiently prolonged duration of peripheral insulin resistance is pivotal for development of cognitive dysfunction. Literature evidence also reveals that impaired cerebral glucose utilization and energy metabolism represent very early abnormalities that precede initial stage of cognitive impairment in AD (Iwangoff et al., 1980; Hoyer, 2004). Since we did not observe any significant difference in memory score between 22nd and 24th week of fructose fed rats, we did not extend our study beyond 24 weeks. This is in contrast to reports of Messier et al. (2007) who did not observe any weight gain or disrupted glucoregulation in response to ad lib access to 15% fructose solution for 4 months in mice and significant an improvement in learning and memory.
Significant strain and species differences exist in susceptibility to impact of fructose-rich diets.

Fructose can uncontrollably produce glucose, glycogen, lactate and pyruvate, providing both the glycerol and acyl portions of acyl-glycerol molecules. These particular substrates, and the resultant excess energy flux due to unregulated fructose metabolism, will promote the overproduction of TG (Mayes, 1993). We also observed that chronic fructose consumption time dependently increased total cholesterol, LDL cholesterol as well as TGs levels and significantly decreased HDL cholesterol levels. Our results are in sync with a previous study of Ohnogi et al. (2012) showing that 15% fructose solution as drinking water for 11 weeks significantly increased the levels of serum TG in male Wistar rats. The Pearson correlation analysis in our study revealed a positive correlation exists between serum TG levels and insulin resistance index, as well as a positive correlation exists between fructose-induced memory deficits and serum triglyceride levels, indicating a strong association of TG with memory functions. Thus the present findings are consistent with a previous report showing that a positive correlation between serum triglyceride and insulin resistance with memory loss in male SD rats (Agrawal and Gomez-Pinilla, 2012). Another study by Farr et al. (2008) demonstrated that injecting TGs directly into the brain ventricles impairs memory possibly via higher apolipoprotein B (apo B) levels.

Further, an increase in the fructose catabolism with the cellular ATP depletion can increase the susceptibility of cells to lipid peroxidation. Moreover, insulin/IGF-1 deficiency leads to increased oxidative stress (de la Monte, 2009). Oxidative stress is reported as one of the earliest events in the pathogenesis of neurodegenerative diseases including AD (Reddy et al., 2009). In the present study, we observed a significant elevation of oxidative damage markers as TBARS, brain nitrite levels and a marked decrease in SOD and catalase activity in the brains of fructose-drinking rats. The generation of reactive nitrogen and oxygen species triggers neuronal damage via microglial activation (Liu et al., 2003). Moreover, oxidative stress seems to affect APP either directly by increasing APP levels or indirectly by modulating APP processing thus increasing the levels of Aβ (Sims-Robinson et al., 2010).
The activation of microglia releases proinflammatory cytokines TNF-α and IL-1β (Merrill and Benveniste, 1996). Various studies have suggested that proinflammatory cytokines, oxygen and nitrogen centered free radicals contribute to the neurodegeneration underlying cognitive deficits in AD (Tanaka et al., 2006). TNF-α and IL-1β have been suggested as important mediators in etiology of AD (Tan et al., 2007). In the present study TNF-α and IL-1β levels started increasing from 7th week and at 24th week, when the memory deficit was at its peak, there was 6.8 fold and 7.5 fold increase in TNF-α and IL-1β respectively. These cytokines can lead to activation of the complement cascade and neuronal damage.

Transforming growth factors have been implicated in the pathogenesis of various neurodegenerative disorders including septic encephalopathy (Semmler et al., 2008), HIV associated dementia and neuroinflammation (Dhar et al., 2006). In the present study, we found 2.8 fold elevated serum TGF-β1 levels at 24th week of fructose drinking in rats. To the best of our knowledge, this is the first report suggesting involvement of TGF-β1 in memory deficits associated with chronic fructose fed rats. TGF β1 and TNF-α are also known to potentiate nitric oxide production in astrocyte cultures by recruiting distinct sub populations of cells to express nitric oxide synthase-2 (Hamby et al., 2008).

Further, to decipher the role of neuronal apoptosis in fructose-induced cognitive dysfunction, we measured NF-κB p65 subunit and caspase-3 levels and observed a significant enhancement in levels of NF-κB and caspase-3 in the cerebral cortex and hippocampus of fructose administered rats. Our findings are supported by results from Liu et al. (2010) who found significant increase in NF-κB activity in fructose-drinking rats, which may contribute to neurological impairment in this animal model of insulin resistance.

Elevated plasma homocysteine (Hcy) levels are associated with cognitive impairments, including age-related cognitive decline and vascular dementia (Troen and Rosenberg, 2005). Case-control studies have shown a significant association between plasma Hcy and insulin levels in human and animal models (El Mesallamy et al., 2010). Hyperhomocysteinemia can induce tau hyperphosphorylation, increase Aβ level in the brain, interrupt DNA
repair in hippocampal neurons and make neurons more vulnerable to the amyloid toxicity (Kruman et al., 2002). Rats fed a fructose-enriched diet have a 72% higher homocysteine levels after 5 weeks compared to chow-fed controls (Oron-Herman et al., 2003). In the present study, we found that fructose drinking rats had 4.83 fold increased homocysteine level as compared to control group at 24\textsuperscript{th} week.

The deficit of cholinergic neurotransmission is an important mechanism in the pathogenesis of AD, and it correlates closely with the severity of cognitive impairment in rats, mice as well as humans with central insulin resistance. The mechanisms underlying the cholinergic deficit induced by insulin resistance in brain still need to be clarified. However, insulin regulates expression of neurotransmitter ACh (Cholerton et al., 2013). In the present study, we found a 3 fold increased AChE activity in fructose-drinking rats. This is consistent with a previous study that AChE activity was increased in some of the brain regions in streptozotocin treated central insulin resistant rats (Salkovic-Petrisic and Hoyer, 2007) as well as in fructose-drinking insulin resistant rats (Luo et al., 2011). Fructose-drinking induces Aβ overproduction and deposition in cortex and hippocampus of rat brain with increased β-secretase-1,γ-secretase-1 activity as well as insulin degrading enzyme activity (Luo et al., 2011) and AChE activity is increased within and around amyloid plaques (Melo et al., 2003). However, AChE was over-expressed in response to various oxidative stresses (Schmatz et al., 2009), thus it is possible that increased AChE activity in hippocampus and cortex, is due to oxidative damage and/or Aβ deposition induced by insulin resistance.

**CONCLUSION**

Conclusively the construction of this time response study reveals that the hallmark characteristics of insulin resistance appear from 7\textsuperscript{th} week of fructose feeding and keep on progressing till 24\textsuperscript{th} week whereas, the cognitive dysfunction appears on 20\textsuperscript{th} week and persists till the end of study. Fructose-induced increase in TG levels; oxidative stress and neuroinflammation all result in abnormal neuronal signaling and synaptic plasticity, thus impairing cognition.