Clinical Nutrition 38 (2019) 1447–1456

Contents lists available at ScienceDirect

Clinical Nutrition

journal homepage: http://www.elsevier.com/locate/clnu

Original article

Time-response studies on development of cognitive deficits in an experimental model of insulin resistance

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ARTICLE INFO

Article history:
Received 22 August 2017
Accepted 19 June 2018

Keywords:
Insulin resistance
Cognitive deficits
Metabolic syndrome
Fructose feeding
Neuroinflammation

SUMMARY

Background & Aims: Alzheimer's disease is suggested to be primarily metabolic, mainly characterized by brain insulin resistance. Chronic fructose feeding results in hippocampal insulin resistance. However, variable opinion exists regarding the concentration and duration of fructose feeding to trigger insulin resistance and resultant cognitive insults. Therefore this study was planned to construct a time–response curve of the appearance of fructose-induced insulin resistance and memory insufficiencies in rats over a period of 24 weeks. Further, Pearson's correlations were drawn between indices of insulin resistance and markers of memory deficits at various time points.

Methods: Male Wistar rats (6 weeks old; 155 ± 5 g) were fed with 15% fructose in normal drinking water for a period of 24 weeks. Body weight, food and water intake were weekly monitored. Fasting blood glucose, glycosylated hemoglobin (HbA_{1C}), lipid profiling, plasma insulin, HOMA-IR index, and systolic blood pressure were estimated to confirm the manifestation of insulin resistance. Cognitive derangements were evaluated by Elevated plus maze and Morris water maze at different time points during the study.

Results: Most of the parameters including insulin resistance became evident at the 7th week and continued until the end of study (24th week) whereas cognitive insufficiency became significantly distinct at the 20th, 22nd and 24th week. Significantly increased serum nitro-oxidative stress, inflammatory cytokines and serum homocysteine levels were intensely connected with fructose-induced neuronal deficits.

Conclusions: The construction of time response study reveals that the hallmark characteristics of insulin resistance appear from the 7th week of fructose feeding whereas the cognitive dysfunction appears on the 20th week and both persist till the end of the study. Fructose-induced oxidative stress and neuroinflammation plausibly impair neuronal signaling and synaptic plasticity.

1. Introduction

Insulin controls glucose uptake, utilization and metabolism in peripheral tissues. It readily diffuses through blood–brain barrier (BBB) via a receptor-facilitated process [1]. Insulin receptors are predominantly available in the synapses of astrocytes and neurons [2]. The insulin receptors including insulin receptor substrate (IRS) and other signaling molecules are expressed in most of the peripheral organs as well as in brain tissue. The rise in peripheral insulin levels is directly linked to elevated insulin levels in brain and cerebrospinal fluid, whereas persistent peripheral hyperinsulinemia is linked to down-regulation of insulin receptors on BBB and diminishes insulin transport into the brain [3].

Existing literature shows that insulin has an important role in physiological hippocampal memory processes [4,5]. Neuronal or brain insulin resistance (B-IR) has been anticipated to be directly connected with the pathological advancement of Alzheimer's disease [6]. Defective insulin signaling in the brain leads to 'an insulin-resistant brain state', sometimes referred to as type-III Diabetes Mellitus [7].

Excessive fructose consumption has become an imperative factor towards the surge in metabolic syndrome cases [8]. Preclinical studies in rats have shown that high-fructose consumption causes hyperinsulinemia, hypertriglyceridemia, hepatic steatosis in

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https://doi.org/10.1016/j.clnu.2018.06.966

addition to endothelial dysfunction [9,10]. In liver, fructose is primarily metabolized to lipids and causes hypertriglyceridemia [11,12]. Triglycerides (TG) in circulation can breach the BBB to give rise to hippocampal insulin resistance, which is believed to cause cognitive insults [13]. Direct injection of TGs into the brain ventricles has shown to impair memory [14]. Evidence from recent research involving chronic fructose feeding amplified fructose metabolizing capacity of neurons [15] and also led to increased expression of hippocampal glucose transporters (GLUT5) [16].

However, an overview of literature provides a variable opinion regarding the fructose concentration and duration of fructose feeding needed to induce insulin resistance and resultant cognitive dysfunction. Previous studies illustrated that acute feeding of fructose leads to improved memory [17]. However, long-term access to fructose induces variable effects on learning and memory [13,18].

Thus the present study aimed to construct a time–response curve of the appearance of insulin resistance and prediction of memory deficits by feeding 15% fructose 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.

2. Material and methods

2.1. Animals

Male Wistar rats (155 ± 5 g) were acquired from Central Animal House facility of Panjab University and accommodated under standard laboratory animal housing environment with 12:12 h light: dark cycle and *ad libitum* access to food (Ashirwad Industries, Mohali, India) and water. Animals were quarantined before initiating the experiment and were familiarized to respective laboratory conditions prior to all the behavioral tests. The use of laboratory animals was permitted by the Institutional Animal Ethics Committee (IAEC/175/UIPS/13 dated 30/8/11) of Panjab University and all experiments were performed as per the guidelines laid by Committee for Control and Supervision of Experimentation on Animals (CPCSEA), Government of India on animal experimentation. All the behavioral tests were carried out during the light phase of the day between 0900 and 1700 h.

A total number of 84 rats were indiscriminately allocated into fourteen diverse groups with 6 animals in each group. Animal groups 1–7 comprised of control rats for each time point, being fed with normal drinking water and chow feed and in case of groups-8-14, normal drinking water was substituted with 15% w/v D-fructose solution along with normal chow for 7, 10, 14, 16, 18, 20, 22 and 24 weeks respectively for development of insulin resistance and associated memory impairment (Fig. 1).

The rats fasted for 8 h from 0900 to 1700 h and blood samples were collected between 1700 and 1900 h, from retro-orbital plexus under Thiopentone sodium-induced anesthesia at different time points i.e. after 7th, 10th 14th, 16th, 18th, 20th, 22nd and 24th week for the assessment of metabolic parameters. Cervical dislocation method was preferred to sacrifice the rats followed by brain isolation, cleaning with ice-cold saline and stored at −80 °C till further analysis.

2.2. Chemicals and kits

D-fructose was procured 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 obtained from R&D Systems, Minneapolis, MN, USA. Insulin and homocysteine ELISA kits were acquired from DRG, Marburg, Germany and Wkea Med Supplies Corp, China respectively. ELISA kits of caspase-3 and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) were procured from Biovision, USA and Imegenex, San Diego, USA respectively. Only analytical grade chemicals were used for biochemical assays.

2.3. Experimental design and treatment protocol

2.3.1. Assessment of physiological parameters

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

Food and water intake was monitored with help of metabolic cages and body weight of the animals were also recorded every week until the end of the experiment. Blood pressure was monitored after 24 weeks of fructose feeding by non-invasive tail cuff method using NIBP instrument (AD Instruments, Australia). Animals were acclimatized for three consecutive days for 20 min a day.

ii. Estimation of hemodynamic parameters

Various hemodynamic parameters were assessed in blood plasma. A) Plasma glucose level was measured using Glucose GOD-POD endpoint kit (ERBA Diagnostics, Mannheim GmbH, Germany). B) Lipid profile including high-density lipoproteins (HDL), low-density lipoproteins (LDL), total cholesterol and triglycerides (TG) was done in blood plasma using diagnostic kits (ERBA Diagnostics, Mannheim GmbH, Germany). C) Percent glycosylated hemoglobin (HbA1c) was measured by cation exchange resin method (Excel Diagnostics, Hyderabad) after 7th, 10th 14th, 16th, 18th, 20th, 22nd and 24th week and the assays were conducted according to the manufacturer's instructions.

2.4. Assessment of behavioral paradigms

The behavioral tests such as morris water maze (MWM) (learning & memory consolidation), elevated plus maze (EPM) (memory retention & acquisition) were performed after 7th, 14th, 16th, 18th, 20th, 22nd and 24th week of the study.

2.4.1. Morris water maze (MWM)

MWM was used for the assessment of spatial memory [20,21]. Animals of various groups were subjected to this test after 7th, 14th, 16th, 18th, 20th, 22nd and 24th week. The maze was a circular water tank with standard measurements (180 cm in diameter and 60 cm high). An unnoticeable platform (12.5 cm in diameter and 38 cm high) was kept 2 cm underneath the water level in one of the four quadrants of the tank. Various brightly colored cues or signs were placed around the maze which is noticeable by rats in the maze, these cues will help the rats in spatial coordination. The locations of the platform and cues continued to be same throughout the study. The experimental environment was spacious, noise free and was maintained at 28.5 ± 2 °C. The MWM task was performed for five consecutive days, out of which rats have given training trials for initial four days; four trials a day with each trial of a maximum time of 90 s (s) with a 30 s interval between each trial. One after another rat was released into the water maze at one of four quadrants with their heads facing the wall of the maze. The rat left for 90 s to find the invisible platform. After reaching onto the platform, the animal was left there for 20 s to observe the surroundings before proceeding with next trial. If the rat failed to reach the platform within the maximally allowed time of 90 s, it was guided with the help of a rod and allowed to remain on the platform for 20 s. Each rat was given four trials a day, each from all the four quadrants and for four consecutive days. The time to reach the platform (escape latency in seconds) and total distance traveled

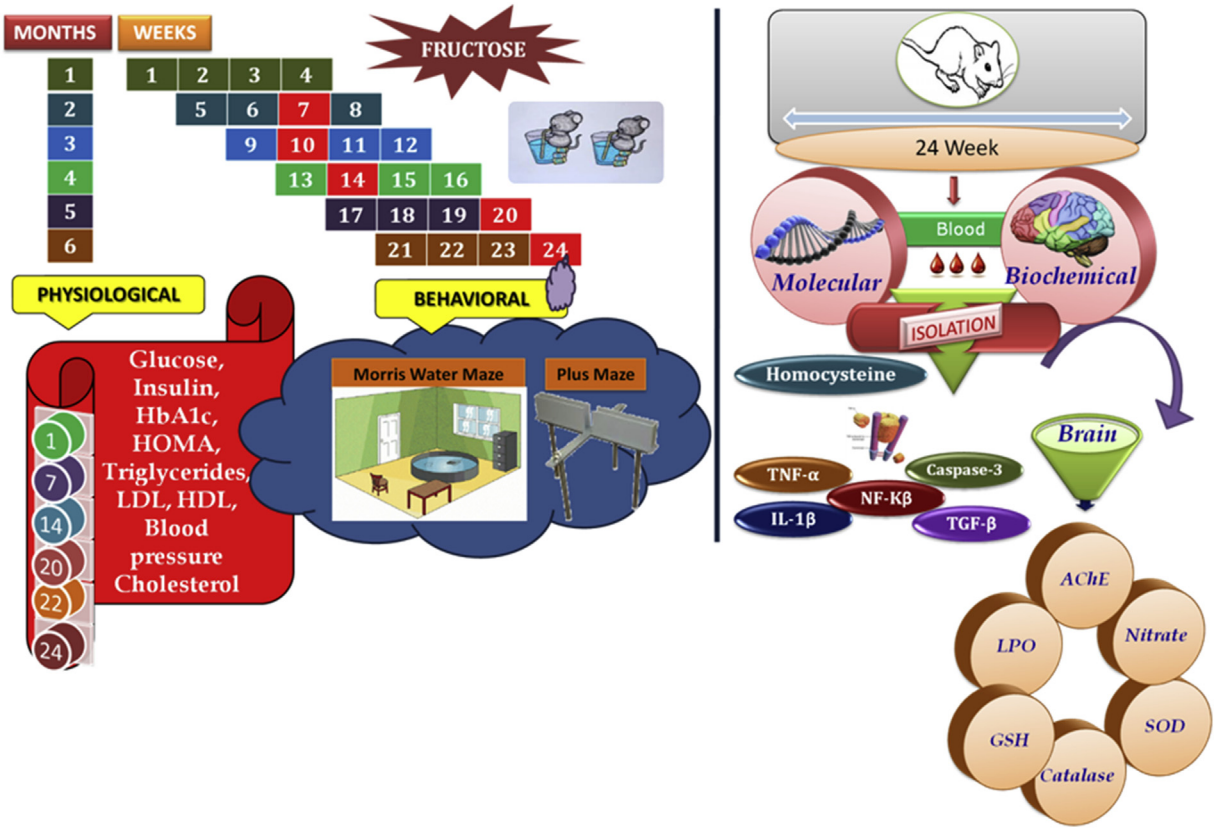

Fig. 1. A schematic diagram of protocol design.

to reach the hidden platform (path length in cm) were measured by using computer tracking system with EthoVision 3.1 version software (Noldus Information Technology, Wageningen, Netherlands).

2.4.2. Memory consolidation test: probe trial

A probe trial was performed [21] on fifth day of MWM test to evaluate the degree of memory consolidation after rats being trained for four days. The hidden platform was removed from the maze and the particular quadrant was then considered as the target quadrant. Each rat was released into water maze for a total duration of the 90s. Total time spent in target quadrant and frequency or number of entries into the target quadrant was calculated with computer-assisted EthoVision software.

2.4.3. Memory acquisition and retention

Animals were tested in EPM to assess the memory acquisition and retention after 7th, 14th, 16th, 18th, 20th, 22nd and 24th week of the study. The EPM comprised of four crossed arms, of which two were open and two closed. One by one, the rats were introduced on to open arm, facing outwards. On the first day (the first trial: Acquisition trial), the time taken by the rat to enter the closed arm was noted as initial transfer latency (ITL). Every trial had a cut-off time of 90 s. If a rat could not reach into the closed arm within cut-off time, it was gently guided into one of the closed arms and permitted to inspect the maze for 30 s. On the next day, a retention trial was performed and the retention trial latency was expressed as a percentage of initial trial latency [22,23].

2.5. Biochemical estimations

Endogenous enzyme activities including acetylcholine esterase, catalase, superoxide dismutase and oxido-nitrosative stress markers such as levels of malondialdehyde, reduced glutathione and nitrites were assessed in cortex and hippocampal regions of the animals sacrificed after 22nd and 24th week.

2.5.1. Brain tissue homogenate preparation

Isolated brains were washed with chilled saline (0.9% sodium chloride) to clear off the blood. Cortex and hippocampus regions were dissected out of the whole brain and these brain regions were homogenized separately in ice-cold phosphate buffer (pH 7.4). The tissue homogenates were first centrifuged (800×*g*/5 min/4 °C) to remove the nuclear debris followed by another step of centrifugation (10,500×*g*/20 min/4 °C) to obtain post-mitochondrial supernatant which was further used for biochemical assays including acetylcholinesterase activity, lipid peroxidation, catalase, nitrite, reduced glutathione and superoxide dismutase activity. The homogenates (post-mitochondrial supernantants) of cortex and hippocampus were stored at −20 °C until further analysis.

2.5.2. Acetylcholinesterase activity

The deregulated cholinergic function was measured by means of acetylcholinesterase activity in homogenates of the cerebral cortex and hippocampal by Ellman's method [24]. The reaction mixture consisted 0.05 mL of postmitochondrial supernatant, 0.10 mL 5,5′-dithiobis (2-nitrobenzoic acid) (Ellman's reagent), 0.10 mL of acetylthiocholine iodide and 3 mL of 0.01 M sodium phosphate buffer (pH 8). The change in optical density was measured at 412 nm for 5 min. The molar extinction coefficient of the chromophore ($1.36 \times 104\ M^{-1}\ cm^{-1}$) was used for the calculations. The results were expressed as a percentage of the control group.

2.5.3. Quantification of lipid peroxidation

Malondialdehyde serves as a marker to assess the extent of lipid peroxidation in tissues. Thiobarbituric acid-reactive substances (TBARS) assay by Wills method [25] was followed. 0.5 mL of post-

mitochondrial supernatant was mixed with equal volume of tris–hydrochloride and incubated at 37 °C for 2 h. 1 mL of 10% trichloroacetic acid (TCA) was added to the reaction mixture and centrifuged (1000×*g*/10 min/room temperature) to separate the supernatant. Equal volumes of supernatant and 0.67% thiobarbituric acid (TBA) were taken into the tubes and kept in boiling water for 10 min. After cooling, an equal volume of double distilled water was added to the supernatant and absorbance was measured at 532 nm. TBARS level was measured using an extinction coefficient of 1.56 × 105 M^{-1} cm^{-1} and expressed as nmol of malondialdehyde per mg protein.

2.5.4. Quantification of reduced glutathione

Glutathione is an endogenous non-enzymatic antioxidant that has the capability of donating an electron to free radical ions. The reduced form of glutathione was estimated by Jollow et al., method [26]. The assay protocol briefly, 1.0 mL of post-mitochondrial supernatant was added to an equal volume of sulphosalicylic acid (4%) to precipitate the samples by incubation at 4 °C for 60 min. Precipitated samples then subjected to centrifugation (1200×*g*/15 min/4 °C) to obtain a clear supernatant. The assay mixture contained 0.1 mL supernatant, 2.7 mL phosphate buffer (0.1 M, pH 7.4) and 0.2 mL Ellman's reagent (0.1 mM, pH 8.0) in a total volume of 3.0 mL. The developed yellow color was read immediately at 412 nm.

2.5.5. Quantification of superoxide dismutase (SOD)

Cytosolic SOD was quantified by Kono method [27]. 2 mL of assay reagent mix (0.1 mM EDTA + 50 mM sodium carbonate + 96 mM of nitro blue tetrazolium (NBT)) was dispensed into a glass cuvette, a mixture of post-mitochondrial supernatant (0.05 mL) and hydroxylamine hydrochloride (0.05 mL; pH 6.0) was added to the cuvette. The levels of SOD were measured in terms of auto-oxidation of hydroxylamine. Changes in optical density were recorded at 560 nm for 2 min at 30/60 s intervals.

2.5.6. Estimation of endogenous catalase

Claiborne method [28] as described in our previous study [29] was followed to estimate brain catalase levels. 0.05 mL of post-mitochondrial supernatant, 1.95 mL phosphate buffer (0.05 M, pH 7.0) and 1.0 mL hydrogen peroxide (0.019 M) in a total volume of 3.0 mL. Variations in optical density were noted at a wavelength of 240 nm and the enzyme activity was expressed in terms of K min^{-1}.

2.5.7. Estimation of nitrosative stress

Nitric oxide (nitrate-nitrite), a byproduct and as well a marker of nitrosative stress and was quantified by a standard total nitric oxide assay kit (Assay Design, Inc. USA). 0.05 mL of brain tissue homogenate was added with nitrate reductase to reduce nitrate to nitrite in presence of nicotinamide adenine dinucleotide 3-phosphate (NADPH). Later, the addition of Griess reagent converts the nitrite into a quantifiable deep purple azo compound. Sodium nitrate solution was used as a standard to calculate the total nitrite/nitrate concentration. Optical density was measured at 540 nm and results were depicted as μmoles/mg protein.

2.6. Assessment of hormones and inflammatory markers

Blood-based assays such as plasma insulin and serum homocysteine, inflammatory cytokines were assessed on 7th, 10th 14th, 16th, 18th, 20th, 22nd and 24th week of the study. Hippocampal inflammatory cytokines were quantified on 22nd and 24th week whereas NF-κB in cortex and hippocampus was measured on the 24th week of the experiment.

2.6.1. Insulin

Plasma insulin levels were measured using a solid phase sandwich enzyme linked immunosorbent assay (ELISA) kit (*DRG, Germany*). 0.025 mL of sample was added to anti-insulin antibodies and the rest procedure was followed as described in the user manual. The absorbance was read at 450 nm. Concentrations of insulin were calculated from plotted standard curves.

2.6.2. Insulin resistance index

Insulin resistance (HOMA-IR) index was calculated by the following formula [19].

$$HOMA = \frac{\text{fasting insulin (mUI/mL) X fasting glucose (mmol/L)}}{22.5}$$

2.6.3. Serum homocysteine (Hcy)

Serum Hcy levels were measured using Rat Hcy ELISA kit (DRG, Germany). The assay was performed using 0.025 mL of serum according to the manufacturer's manual. The plate was incubated after addition of every reagent which varied from 15 to 30 min and the absorbance was noted at 450 nm.

2.6.4. Serum TNF-α, TGF-β1 and IL-1β

The quantification of TNF-α, TGF-β1 and IL-1β in rat serum were done with Quantikine Rat IL-1β, TGF-β1 and TNF-α ELISA kits (R&D Systems, USA). The assay was done using 0.1 mL of serum according to the manufacturer's instructions.

2.6.5. Hippocampal TNF-α, TGF-β1 and IL-1β

The quantification of TNF-α, TGF-β1 and Il-1β in rat hippocampal were done with Quantikine Rat IL-1β, TGF-β1 and TNF-α ELISA kits (R&D Systems, USA). The assay was carried out using 0.1 mL in accordance with manufacturer's procedure.

2.6.6. Estimation of NF-κB p65 unit

The levels of p65 in post-mitochondrial supernatant fractions of cortex and hippocampus can be positively associated with stimulation of NF-κB pathway. NF-κB/p65 unit was assessed by Activ ELISA (Imgenex, San Diego, USA) kit. The assay was done using 0.1 mL according to the manufacturer's instructions. The results were shown as ng/mg protein.

2.6.7. Quantification of Caspase-3

Caspase-3 enzyme levels in cytosolic fractions of cortex and hippocampus were estimated by caspase-3 colorimetric kit (R&D systems, USA) according to the manufacturer's manual. The assay protocol briefly, 0.05 mL of sample was added to an equal amount of reaction buffer containing 1% DTT and 0.005 mL of Caspase-3 colorimetric substrate (DEVD-pNA) added followed by incubation at 37 °C for 2 h.The absorbance was read at 405 nm. Caspase-3 levels were depicted as ng/mg protein.

2.7. Statistical analysis

GraphPad Prism® statistical software version 5.01 was used to analyze the data and all the results were represented as mean ± S.E.M. Intergroup variance for most of the parameters was calculated by one-way analysis of variance (ANOVA) followed by Tukey's test except the MWM parameters, where two-way ANOVA followed by Tukey's test was employed. A value of $p < 0.05$ was regarded statistically significant.

3. Results

3.1. Effect of fructose on basic physiological parameters

Fructose-fed animals exhibited increased fasting plasma glucose, glycosylated hemoglobin and fasting plasma insulin 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 a marked escalation in body weight besides altered lipid profile in a time-dependent manner. Systolic blood pressure was significantly increased at 24 weeks in fructose-drinking rats (164.85 mm Hg ± 3.19) as compared to control (117.83 mm Hg ± 3.97). These observations clearly demonstrated marked alterations in physiological parameters due to chronic fructose consumption (Table 1).

3.2. Behavioral interpretations

3.2.1. Effect of fructose on performance in MWM task

The cognitive functions include task learning was evaluated in the MWM test. On day 1, the mean escape latency was almost same in control and fructose-fed animal groups but it started varying from the second day. Although, there was no significant change in escape latency was observed in 7th, 14th, 16th and 18th-week groups but a significant increase in escape latency was observed after 20th, 22nd and 24th week. Rats showed poorer learning ability with significantly reduced performance to find out the platform in the probe trial after the 20th week of fructose (15%w/v) administration which was persisted until the end of the study (Fig. 2A).

3.2.2. Effect of fructose on path length to find the hidden platform

In the water maze test, the abridged path length to find the hidden platform on successive days of training is linked to efficient learning and intact memory of rats. On day 1, the total distance traveled to locate the hidden platform was almost similar in both fructose-fed groups and normal control. There was a significant increase in path length in probe trial at 20th, 22nd and 24th week of fructose-fed animals as compared to control group (Fig. 2B). However, no significant change was witnessed in path length in probe trial between control and fructose group at 7th, 14th, 16th, 18th week.

3.2.3. Effect of fructose on time spent in the target quadrant

The probe trial indicates the learning proficiency and the ability to recollect the location of the hidden platform during the four-day training. Rats of control and fructose-fed groups showed no difference after 7th, 14th, 16th and 18th week of fructose feeding but after 20th, 22nd and 24th week, a significant difference was observed. The time spent in the target quadrant was significantly lowered after 20 weeks in fructose-fed rats as compared to the control group (Fig. 2C).

3.2.4. Effect of fructose feeding on the frequency of appearance in the target quadrant

During the probe trial or 90 s, the frequency of entries into target quadrant was significantly decreased on 20th, 22nd and 24th week of fructose feeding as compared to control group. However, there was no significant change was observed between normal control and fructose-fed groups at 7th, 14th, 16th and 18th week (Fig. 2D).

3.2.5. Effect of fructose on initial transfer latency in EPM

The changes in initial transfer latency were insignificant in normal control and fructose-fed groups after 7th, 14th, 16th and 18th weeks of fructose administration. However, a significant difference was observed after the 20th, 22nd and 24th week of fructose consumption (Fig. 3A).

3.2.6. Effect of fructose feeding on locomotion

The spontaneous locomotor activity of fructose-fed animals was slightly carried but not significant compared to the control throughout the 24 weeks of study (Fig. 3B).

3.3. Biochemical and molecular estimations

3.3.1. Effect of fructose on acetylcholinesterase activity

Acetylcholinesterase levels were significantly elevated in cerebral cortex and hippocampus after 22 weeks of fructose feeding as compared to normal control group (Table 2).

3.3.2. Effect of fructose on lipid peroxidation

Significantly increased TBARS levels reflected elevated lipid peroxidation in cerebral cortex and hippocampus of fructose-fed

Table 1
Effect of fructose on basic physiological parameters. Values are expressed as mean ± SEM. Body weight, water intake, food intake, plasma glucose, insulin, Hb1Ac, HOMA-index, HDL, LDL, TG and total cholesterol. a indicates change from respective control group; b indicates difference from fructose 7th week; c indicates difference from fructose 10th week; d indicates difference from fructose 14th week; e indicates difference from fructose 16th week; f indicates difference from fructose 18th week; g indicates difference from fructose 20th week; h indicates difference from fructose 22nd week.

	B.Wt (g)	Food intake (gm)	Water intake (mL)	Glucose (mg/dL)	Insulin (pmol/l)	HbA_{1C} (%)	HOMA-IR Index	HDL (mg/dL)	LDL (mg/dL)	Cholesterol (mg/dL)	Triglycerides (mg/dL)
C-1	155.2 ± 1.0	26.4 ± 0.5	7.2 ± 0.4	97.8 ± 1.2	97.2 ± 0.73	4.5 ± 0.52	3.4 ± 0.1	39.9 ± 1.5	15.1 ± 2.3	62.3 ± 2.7	36.0 ± 1.5
FR-1	157.4 ± 2.2	27.1 ± 1.1	7.4 ± 1.3	97.6 ± 2.1	99.2 ± 1.2	4.7 ± 1.0	3.4 ± 0.1	42.2 ± 2.3	16.3 ± 2.6	63.9 ± 2.5	34.5 ± 3.2
C-7	256.8 ± 4.6	26.4 ± 0.6	7.2 ± 0.8	99.3 ± 2.3	98.3 ± 1.2	4.6 ± 0.8	3.2 ± 0.1	39.4 ± 2.5	14.5 ± 2.6	62.6 ± 3.8	41.3 ± 2.5
FR-7	306.8 ± 3.1^{a}	25 ± 0.7	25.6 ± 0.5^{a}	130.0 ± 2.1^{a}	143.2 ± 7.9^{a}	5.7 ± 0.1^{a}	6.6 ± 0.3^{a}	35.6 ± 0.4^{a}	40.8 ± 2.2^{a}	86.4 ± 1.9^{a}	49.8 ± 1.0^{a}
C-10	308.2 ± 4.5	25.3 ± 0.1	7.1 ± 0.6	99.8 ± 2.4	99.2 ± 0.8	4.7 ± 0.6	3.4 ± 0.1	44.1 ± 3.1	16.1 ± 3.2	61.4 ± 3.6	36.6 ± 2.2
FR-10	371.6 ± 5.7^{a,b}	24.2 ± 0.8	26.6 ± 0.7^{a}	160.2 ± 1.5^{a,b}	190.8 ± 3.9^{a,b}	7.4 ± 0.4^{a,b}	10.9 ± 0.3^{a,b}	34.6 ± 0.6^{a}	53.9 ± 3.2^{a,b}	103.4 ± 2.9^{a,b}	74.4 ± 0.7^{a,b}
C-14	321.6 ± 7.2	24.9 ± 0.4	7.1 ± 0.9	96.9 ± 1.3	101.2 ± 3.1	4.4 ± 0.8	3.4 ± 0.1	41.1 ± 2.2	16.1 ± 3.3	61.7 ± 3.6	36.2 ± 2.5
FR-14	396.8 ± 3.3^{a,c}	21.4 ± 0.7^{a}	51.0 ± 1.9^{a,c}	195.6 ± 2.6^{a,c}	235.2 ± 4.2^{a,c}	7.8 ± 0.2^{a}	16.4 ± 0.5^{a,c}	33.4 ± 0.8^{a}	95.2 ± 6.2^{a,c}	145.5 ± 8.0^{a,c}	84.7 ± 4.8^{a,c}
C-16	339.4 ± 6.2	25.1 ± 0.7	6.7 ± 0.6	97.0 ± 2.3	100.2 ± 0.9	4.7 ± 0.4	3.6 ± 0.1	40.4 ± 2.5	17.0 ± 3.7	61.6 ± 3.2	37.3 ± 2.9
FR-16	428.4 ± 5.0^{a,d}	21.8 ± 0.9^{a}	55.2 ± 0.9^{a,b}	217.8 ± 2.4^{a,e}	256.8 ± 3.9^{a,d}	8.4 ± 0.1^{a}	19.9 ± 0.5^{a,d}	32.3 ± 0.6^{a,d}	106.8 ± 2.9^{a,d}	155.3 ± 2.8^{a,d}	81.1 ± 1.9^{a,c}
C-18	344.6 ± 4.9	24.1 ± 0.8	7.7 ± 1.1	97.2 ± 1.2	99.5 ± 0.8	4.7 ± 0.9	3.3 ± 0.1	39.6 ± 1.2	16.6 ± 2.7	62.7 ± 2.7	36.7 ± 3.7
FR-18	447.6 ± 1.3^{a,e}	22 ± 0.7	53.8 ± 0.9^{a,b}	242 ± 4.7^{a,e}	316.6 ± 4.1^{a,e}	9.6 ± 0.1^{a,d}	27.3 ± 0.5^{a,e}	31.0 ± 0.8^{a,d}	130.4 ± 3.5^{a,e}	181.1 ± 4.0^{a,e}	98.2 ± 1.2^{a,e}
C-20	351.1 ± 4.1	25.1 ± 0.2	7.3 ± 0.4	97.4 ± 2.2	95.4 ± 1.2	5.0 ± 0.8	3.5 ± 0.1	38.2 ± 3.1	16.2 ± 3.4	62.8 ± 2.6	36.4 ± 2.6
FR-20	467.2 ± 2.1^{a,f}	22.4 ± 0.4^{a}	57.2 ± 3.4^{a,d}	251.2 ± 3.0^{a,f}	338.8 ± 1.5^{a,f}	14.8 ± 2.0^{a,f}	30.3 ± 0.2^{a,f}	30.8 ± 0.8^{a,d}	158.1 ± 8.8^{a,f}	214.0 ± 9.0^{a,f}	125.1 ± 5.2^{a,f}
C-22	354.7 ± 6.4	23.9 ± 1.2	7.4 ± 0.4	97.9 ± 2.2	98.8 ± 0.7	4.7 ± 0.5	3.4 ± 0.1	38.4 ± 2.6	16.1 ± 3.5	62.3 ± 2.8	36.9 ± 1.6
FR-22	499.6 ± 7.5^{a,g}	22.2 ± 0.6	62.0 ± 3.1^{a,f}	281.6 ± 3.0^{a,g}	387.4 ± 2.4^{a,g}	14.9 ± 0.2^{a,f}	38.8 ± 0.4^{a,g}	27.2 ± 0.6^{a,g}	188.7 ± 2.1^{a,g}	241.4 ± 1.9^{a,g}	127.2 ± 2.4^{a,f}
C-24	359.2 ± 5.3	24.4 ± 0.5	7.1 ± 0.3	99.1 ± 1.6	98.1 ± 0.4	5.02 ± 1.3	3.4 ± 0.1	39.6 ± 1.4	16.1 ± 3.3	61.2 ± 3.6	36.4 ± 1.9
FR-24	514 ± 6.9^{a,h}	21.6 ± 0.4^{a}	52.0 ± 5.4^{a}	293.2 ± 1.8^{a,h}	414.4 ± 3.7^{a,h}	15.6 ± 0.4^{a,f}	43.2 ± 0.14^{a,h}	25.7 ± 2.33^{a,g}	186.6 ± 8.0^{a,h}	240.2 ± 8.4^{a,g}	136.7 ± 4.8^{a,h}

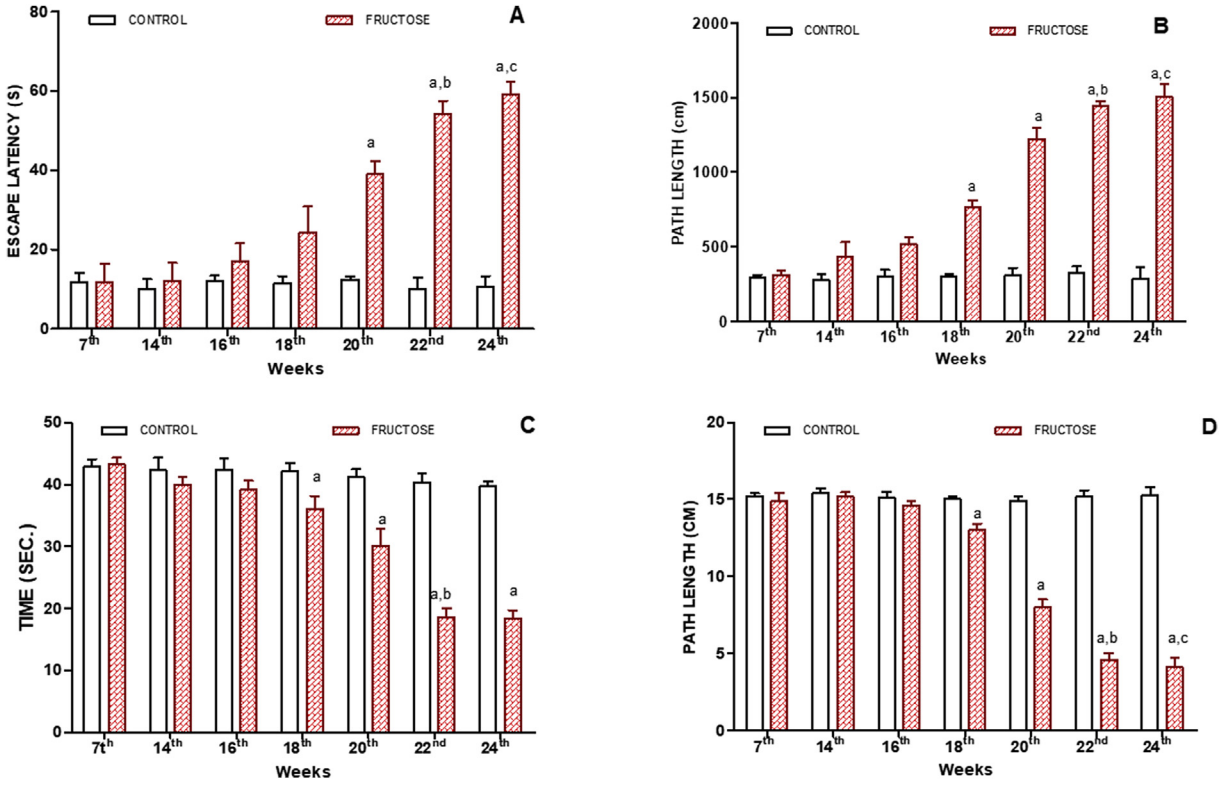

Fig. 2. Effect of fructose feeding at 7th, 14th, 16th, 18th, 20th, 22nd and 24th week on escape latency (A), path length (B) in MWM on day 5. A statistically significant difference between days and group was observed. Time spent in the target quadrant (C) and frequency of appearance in the target quadrant (D) in probe trial. 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 a change from respective control group; b represents the difference from fructose 20th week; c represents the difference from fructose 22nd week.

rats as compared to normal control (Table 2). There was a statistically considerable variation among the mean values of control and fructose group in cerebral cortex and hippocampus. Moreover, lipid peroxidation in selected brain regions was significant between 22nd and 24th weeks.

3.3.3. *Effect of fructose on nitrosative stress in brain*

Total nitric oxide levels in cerebral cortex and hippocampus of fructose-fed rats were significantly increased as compared to control group animals (Table 2).

3.3.4. *Effect of fructose on antioxidant enzymes*

Levels of endogenous antioxidant enzymes such as reduced glutathione, superoxide dismutase and catalase were significantly decreased in cerebral cortex and hippocampus of fructose-fed rats as compared to control group (Table 2).

3.3.5. *Effect of fructose on serum homocysteine, IL-1β, TGF-β and TNF-α levels*

Rats subjected to fructose feeding showed a significant and time-dependent increase in serum homocysteine, TNF-α, TGF-β

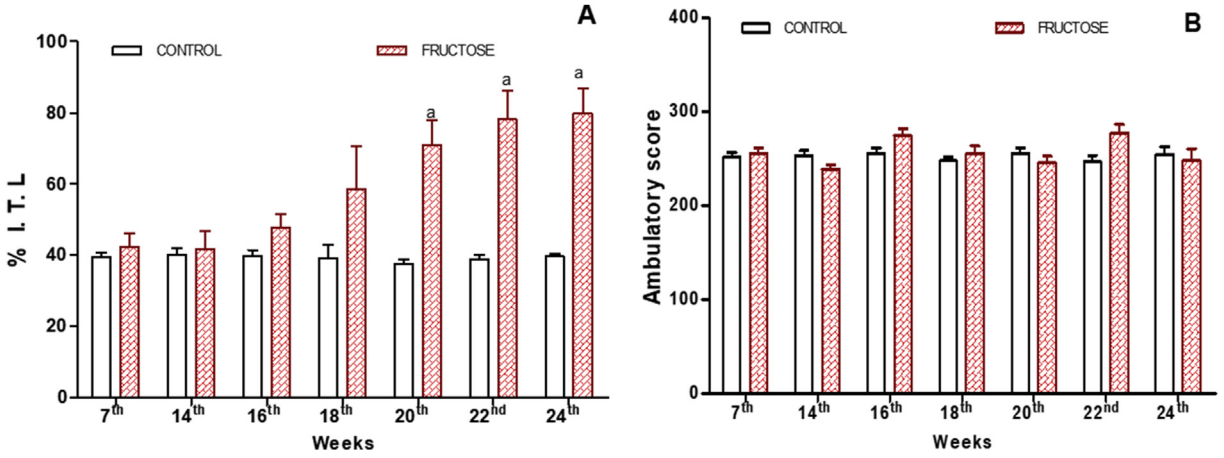

Fig. 3. Effect of fructose feeding at 7th, 14th, 16th, 18th, 20th, 22nd and 24th week on percent initial transfer latency in EPM (A); and ambulatory score in actophotometer (B). Values were represented as mean ± SEM. 7th = 7th week; 14th = 14th week; 16th = 16th week; 18th = 18th week; 20th = 20th week; 22nd = 22nd week; 24th = 24th week. a represents the difference from the respective control group.

Table 2
Consequence of fructose administration on lipid peroxides, glutathione, superoxide dismutase, catalase, nitrite and acetylcholinesterase (AChE) activity, in cerebral cortex and hippocampus. Results 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.

Groups	Brain region	LPO (nmoles/mg protein)	GSH (μmoles/mg protein)	SOD (Units/mg protein)	Catalase (μM of H_2O_2/min/mg protein)	Nitrite (μg/ml)	AChE (μM/min/mg protein)
Control-22	Hippo	1.79 ± 0.014	0.29 ± 0.012	5.50 ± 0.37	11.99 ± 0.74	181.32 ± 15.19	0.012 ± 0.01
	Cortex	1.65 ± 0.02	0.283 ± 0.02	5.69 ± 0.36	12.65 ± 0.57	185.43 ± 12.42	0.014 ± 0.012
FR-22	Hippo	6.25 ± 0.03[a]	1.0 6 ± 0.01[a]	0.64 ± 0.17[a]	2.42 ± 0.72[a]	853.56 ± 31.27[a]	0.04 ± 0.01[a]
	Cortex	5.66 ± 0.022[a]	0.06 ± 0.012[a]	0.69 ± 0.15[a]	2.44 ± 0.53[a]	849.47 ± 32.86[a]	0.033 ± 0.012[a]
Control-24	Hippo	1.71 ± 0.01	0.28 ± 0.015	5.55 ± 0.31	12.07 ± 0.71	182.34 ± 12.14	0.013 ± 0.01
	Cortex	1.67 ± 0.01	0.29 ± 0.01	5.61 ± 0.31	12.61 ± 0.51	181.41 ± 11.49	0.02 ± 0.01
FR-24	Hippo	6.16 ± 0.024[a]	0.0 55 ± 0.012[a]	0.67 ± 0.12[a]	2.46 ± 0.74[a]	869.59 ± 32.53[a]	0.034 ± 0.001[a]
	Cortex	5.89 ± 0.01[a]	0.06 ± 0.01[a]	0.63 ± 0.14[a]	2.52 ± 0.55[a]	899.33 ± 34.73[a]	0.03 ± 0.001[a]

and IL-1β level as compared with control group after 7th, 10th, 14th, 16th, 18th, 20th, 22nd and 24th week of fructose drinking (Fig. 4).

3.3.6. *Effect of 24 weeks fructose feeding on hippocampal pro-inflammatory cytokines*

Twenty four weeks of oral fructose feeding led to a significant rise in rat's hippocampal pro-inflammatory markers such as TNF-α, TGF-β and IL-1β compared to control group (Table 3).

3.3.7. *Effect of fructose consumption on NF-κB and caspase-3 activity*

We observed 2.4 and 2.9 fold increase NF-κβ p65 subunit expression in cerebral cortex and hippocampus respectively after 24 weeks in fructose-fed rats. In addition, caspase-3 activity was 3.5 fold increase in the cerebral cortex and 3.7 fold increase in the hippocampus after 24 weeks of fructose administration (Fig. 5).

3.3.8. *Correlation between elevated triglycerides levels and cognitive function*

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

3.3.9. *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

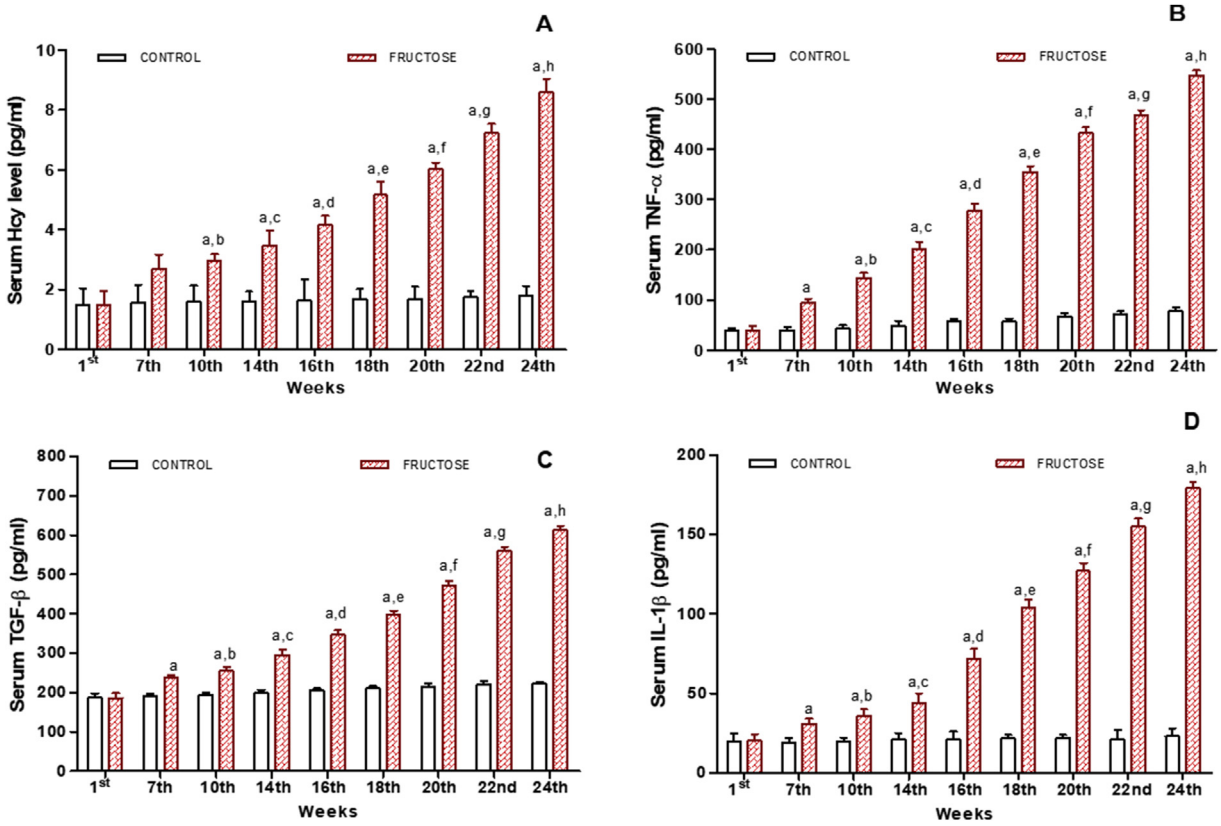

Fig. 4. Influence of fructose administration on serum Hcy (A), TNF-α level (B), TGF-β level (C) and IL-1β level (D) Values were expressed as mean ± SEM. 1st = 1st week; 7th = 7th week; 10th = 10th week; 14th = 14th week; 16th = 16th week; 18th = 18th week; 20th = 20th week; 22nd = 22nd week; 24th = 24th week. a represents difference from respective control group; b indicates change from fructose 7th week; c indicates change from fructose 10th week; d indicates change from fructose 14th week; e indicates change from fructose 16th week; f indicates change from fructose 18th week; g indicates change from fructose 20th week; h indicates change from fructose 22nd week.

Table 3
Effect of fructose administration on the hippocampal TNF-α level, TGF-β level and IL-1β level on 24th weeks. Values were expressed as mean ± SEM. a represents the difference from control.

Groups	Hippocampal TNF-α	Hippocampal TGF-β	Hippocampal IL-1β
Control	75.3 ± 3.52	205.7 ± 8.97	22.1 ± 3.52
Fructose 24 weeks	335.4 ± 4.57[a]	662.4 ± 7.18[a]	79.2 ± 5.11[a]

24th week of fructose feeding, implying that cognitive function relies on the levels of insulin resistance index (Supplementary data).

4. Discussion

It is being progressively recognized that anomalous glucose metabolism in peripheral and brain tissues can disrupt cognitive functions. For the first time, the present study 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, oral feeding of 15% fructose solution 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 week during the study.

According to Hwang and colleagues, high fructose feeding caused insulin resistance, hyperinsulinemia, hypertriglyceridemia and hypertension in rats [9,10]. In our study, parameters of insulin resistance i.e. body weight, blood glucose, deranged lipid profile, glycosylated hemoglobin, systolic blood pressure, plasma insulin and HOMA-IR index became significantly elevated at 7th week of fructose feeding and kept on increasing till 24 weeks. However, the mean escape latency and path length to reach the hidden platform in MWM 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 the 7th week of fructose feeding but the decline in spatial memory appeared on the 20th week. These observations reveal that sufficiently prolonged duration of peripheral insulin resistance is pivotal for the development of cognitive dysfunction. 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. [17] who did not observe any weight gain or disrupted glucose regulation in response to *ad libitum* access to 15% fructose solution for 16 weeks in mice and significant an improvement in learning and memory. The susceptibility to fructose-rich diets may significantly vary depending upon the species and strain of the animals used.

Dysregulated fructose metabolism can produce glucose, glycogen, lactate and pyruvate besides yielding triglycerides and excessive intake of fructose, uncontrolled fructose breakdown will lead to the disproportionate production of TG [30]. 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. Findings of our study are similar to the previous study of Ohnogi, Hayami [31] showing that 15% w/v fructose solution for 11 weeks considerably elevated the serum TG levels in male Wistar rats. The Pearson correlation analysis in our study has shown an affirmative relationship between increased serum TG levels and insulin resistance index, as well as a constructive association between increased serum TG levels and fructose feeding associated memory deficits, demonstrating a resilient connotation of serum TG with impaired memory functions and similar to the previous reports by Agrawal et al., [32]. Another study by Farr and colleagues [14] demonstrated that intracerebroventricular (ICV) injection of TGs caused memory loss, possibly via higher apolipoprotein B (Apo B) levels.

Further, an increase in the fructose catabolism with reduced cellular ATP can escalate the vulnerability of tissues to lipid peroxidation. Additionally, insulin/IGF-1 insufficiency leads to augmented cellular oxidative stress [33]. Oxidative stress has been described to be one of the initial proceedings in the development of various neurodegenerative diseases including AD [34]. In our study, we observed a significant rise in oxidative stress markers as TBARS, brain nitrite levels and a marked decrease in SOD, reduced glutathione and catalase enzyme levels in the brains of fructose-fed animals. Increased production of reactive nitrogen and oxygen free radicals elicits neuronal destruction via microglial activation [35]. Moreover, oxidative stress may directly interfere with APP production or indirectly by modulating APP processing thus increasing the levels of Aβ [36]. The release of proinflammatory cytokines such as TNF-α and IL-1β is triggered by microglial activation [37]. Various studies on neurodegenerative disorders have suggested that proinflammatory cytokines, reactive oxygen and nitrogen species encourage the damage to neurons [38]. Recruitment of TNF-α and IL-1β have been posted as important mediators in etiology of AD [39]. In our study, we found that the levels of TNF-α and IL-1β begun to rise from the 7th week and at 24th week, when the memory deficit was at its peak, there was 6.8 fold and 7.5 fold surge in TNF-α and IL-1β levels respectively. These cytokines can activate the complement cascades that can lead to neuronal damage.

Transforming growth factors (TGF-β1) involvement in the pathogenesis of various diseases such as septic encephalopathy [40], human immunodeficiency virus (HIV) linked neuroinflammation and dementia [41]. In our study, we found 2.8 fold elevated serum TGF-β1 levels at the 24th week of 15% fructose-fed rats compared to control animals and to the best of our knowledge, results of our study the first ever to report the involvement of TGF-β1 in decreased memory functions associated with chronic fructose feeding. TGF-β1 and TNF-α were well-known for their ability to

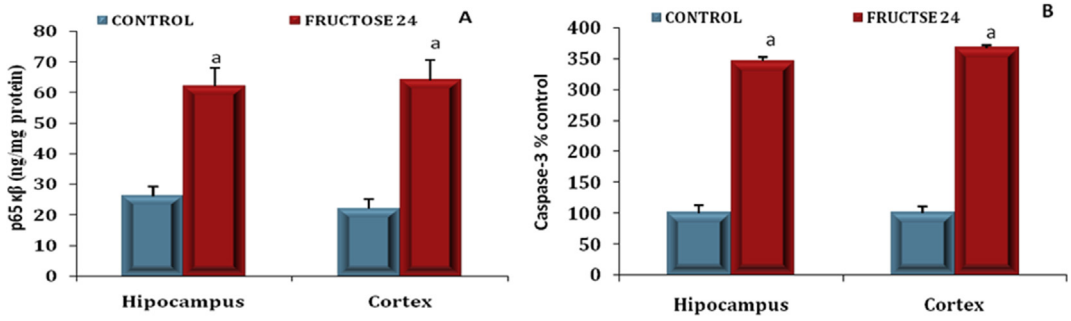

Fig. 5. Effect of fructose drinking on NF-κβ (A) and caspase-3 activity (B). Results were expressed as mean ± SEM and compared to control group. a represents the level of significance at $p < 0.05$ compared to normal control.

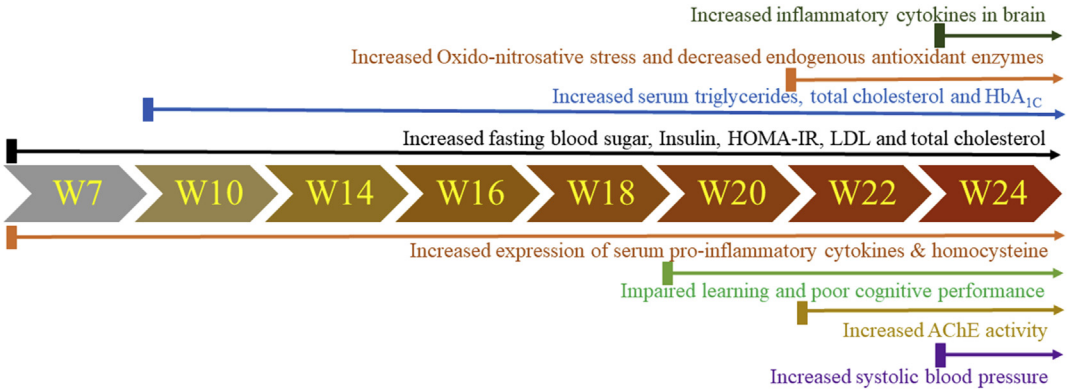

Fig. 6. Time dependent alterations in metabolic, hemodynamic, oxidative stress, inflammatory and cognitive parameters in fructose-fed rats till the 24th week.

stimulate the production of nitric oxide in astrocyte cultures through activation of inducible nitric oxide synthase (iNOS or NOS-2) [42].

Further, we measured the levels NF-κB p65 subunit and caspase-3 to decipher the role of neuronal apoptosis in cognitive deficits associated with oral fructose feeding and we observed a significant escalation in levels of NF-κB and caspase-3 in the cerebral cortex and hippocampus of fructose-fed rats. Our findings are supported by a study done by Liu et al. [43], which reported a significant rise in NF-κB activity in fructose-fed rats, which is anticipated to contribute towards the neurological derangements in our study.

Increased plasma homocysteine (Hcy) levels are linked with vascular dementia and cognitive impairments, including age-related cognitive decline [44]. A significant positive correlation between plasma Hcy and insulin levels were reported by various studies that involved clinical and preclinical models [45]. Elevated Hcy levels in the blood can stimulate hyperphosphorylation of tau, intensification of Aβ level in the brain, interrupt DNA repair in the neurons hippocampus and make the neurons even more susceptible to the amyloid toxicity [46]. Fructose enrich diet fed rats have shown 72 percent higher homocysteine levels after 5 weeks compared to normal chow-fed controls [47]. In our experiment, we have observed a 4.83 fold increased serum homocysteine level as compared to normal pellet chow group at 24th week.

Altered cholinergic neurotransmission is one of the important mechanisms involved in the advancement of Alzheimer's and it is closely related to the brain insulin resistance induced cognitive deformities in preclinical models as well as humans. Though the underlying mechanisms of brain insulin resistance induced cholinergic deficit still unclear. But insulin is known to regulate the expression of ACh [48]. In our study, we found a 3 fold increase in AChE activity in fructose-fed group compared normal rats, that is also corroborated by a previous report demonstrating increased AChE activity in few of the brain areas in Streptozotocin (STZ) induced rat model of brain insulin resistance [49] as well as in fructose-drinking insulin resistant rats [19]. Fructose-drinking causes overproduction and deposition of Aβ in cerebral cortex and hippocampus of rat brain with elevated activities of enzymes such as β-secretase-1, γ-secretase-1, insulin-degrading enzyme [19] and AChE [50]. However, AChE was found to be over-expressed in oxidative stress conditions [51], thus the elevated oxidative stress and or Aβ deposition may be responsible for the increased levels of AChE levels in hippocampus and cortex (Fig. 6).

5. Conclusion

The construction of this time response study reveals that the hallmark characteristics of insulin resistance started to appear from the 7th week of fructose feeding and kept on progressing till 24th week whereas, the cognitive dysfunction was first evident on 20th week and persists till the end of the study. Fructose feeding led to an elevation in TG levels, oxidative stress and neuroinflammation all result in abnormal neuronal signaling and synaptic plasticity, thus impairing cognition.

Author contributions

AKS and KC contributed to study design, data collection and analysis, and RND wrote and revised the manuscript.

Conflict of interest

None declared.

Acknowledgment

The authors would like to acknowledge Council of Scientific and Industrial Research (CSIR), Ministry of Science & Technology (Grant: 09/135/0654/2012- EMR-I), India for providing a Senior Research Fellowship to Dr. Anand Kamal Sachdeva and University Grants Commission (UGC) (Grant: Kanwaljit Chopra, PI UGC-MRP, 39-156/2010), India for funding this work.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.clnu.2018.06.966.

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