INTRODUCTION

Diabetes mellitus with onset in childhood represents one of the most frequent chronic diseases in children as well as in young adults and is characterized by hyperglycaemia and glycosuria resulting from defects in insulin secretion, insulin action, or both.1 In 2010, about 4,80,000 children worldwide were estimated to have type-1 diabetes.2 It is estimated that an annual basis about 76,000 children aged up to 14 years develop type-1 diabetes. Despite, the extremely wide global variation in incidence, the epidemiology of childhood type-1 diabetes has shown increasing over the last few decades of the twentieth century.7 Incidence increased in most countries and this trend was accompanied by a shift in the age of onset to the youngest age group (0-4 years).8 In India, the type of diabetes differs considerably from that in the Western world and type-1 is considerably in fewer rates, while the prevalence of diabetes has shown increasing in the last three decades8. There is anecdotal evidence that the affected children, especially girls tend to be shorter and many of them have delayed puberty. There is ample evidence in literature that subjects with type-1 diabetes have multi-system sensitivity including renal, cardiac and ophthalmic and CNS involvement9. A considerable percentage of children, who are currently being diagnosed with type-1 diabetes have been shown to suffer from neuro-cognitive impairments8. Excessively produced free radicals have been implicated in long duration diabetic children. Studies of Betteridge7 reported that an imbalance in the generation and scavenging of free radicals play an important role in determining tissue damage associated with diabetes. Many studies have also shown a relationship between cognitive dysfunction and type-1 diabetes of early onset. In particular, patients with diabetes onset before the age of 5-years could be more sensitive to the effects of hypoglycemic episodes or elevated HbA1c levels9. Therefore a relationship between cognitive dysfunction and type-1 diabetes of early onset has been established. The question that still remained unresolved is contribution of different disease variables, such as diabetes duration, levels of glycemic control and the changes in hippocampal neuronal circuitry. Therefore the extent to which oxidative stress cause damage to hippocampal neuro circuitry in long duration diabetes that impedes the functional ability of animal especially on cognitive functions. Hence, the present study was aimed at exploring cognitive functions in rats suffering hyperglycaemic complications for long duration.

MATERIALS AND METHODS

Chemicals
All analytical grade chemicals were procured from BDH and Sigma– Aldrich.

Animals
Albino rats, Wistar strain (Rattus norvegicus albinus) of age group (21-day old), were procured from Sri Raghavendra enterprises, Bangalore, India and acclimatized to laboratory conditions (12 h dark/light cycle at 28 ± 2°C) for 1 week prior to commencement of the experiment. They were maintained along with mother rat on standard rodent pellet and tap water ad libitum; in accordance with the guidelines of National Institute of Nutrition, ICMR, Hyderabad, India and experimental protocol was approved by the Institutional animal ethical committee, Bangalore University, Bangalore, India (CPCSEA No.402, File No.25/525/2009 dated 23.03. 2011).

Design

Induction of Diabetes
Animals were maintained for four months as per the approved protocol of the study and alloxan (200 mg/kg b wt/week for 3-weeks) was administered intra-peritoneally to
induce diabetes (n = 48). Blood samples from tail vein puncture following overnight fasting were measured to confirm the alloxan induced diabetic state. Rats, which were able to achieve the required diabetic state (FBS more than 200 mg % measured with Standard Glucometer (One-Touch Horizon, Life Scan, a division of Johnson and Johnson Pvt. Ltd product, India) after alloxan injection were only included in the study and the day of confirmation of diabetes was considered as day one of diabetic state. Blood glucose levels were monitored weekly throughout the period of study. Behavioural and biochemical assessments were made upon 1, 2, 3 and 4-months duration of diabetes.

**Behavioural Assessments**

**T-maze**

The T-maze consisted of a start box (12 x 12 cm), a stem (35 x 12 cm), a choice area (15 x 12 cm) and two arms (35 x 12 cm) at the end of which was the goal area (15 x 12 cm) containing the food well. The sidewalls were of 40 cm height. The stem and the start box were separated by a sliding door as depicted in the schematic diagram. A cloth curtain covered the goal area from the arm so that the animal cannot see the food well from the choice area. The T-maze was kept in a dimly lit, sound attenuated room, two feet above the ground level.

**Rewarded alternation task**

After 48 h of semi starvation, all experimental and control group of rats were assessed for the acquisition and performance of rewarded alternation task in a T-maze. The test consisted of an orientation session of 30 minutes duration, followed by test sessions. On the first day of orientation session, the rats were placed in T-maze for 30 minutes for orientation and familiarization of the T-maze environment. The food pellets were provided, whenever the rat reached the goal area. After the orientation session, they were subjected to rewarded alternation task consisting of 10 trials per session/day. In the first trial the rat was placed in the start box, sliding door was released slowly and the rat was allowed to move out and enter into the stem and then any arm of the T-maze. Once the rat reaches the goal area and eats the food pellet, it was removed and placed in the home cage. After 30 seconds of inter trial interval (during this period the arms were quickly and thoroughly cleaned with 70% alcohol), the rat was again placed back in the start box for the second trial. The food pellet was placed alternately in the right or left goal areas, so that the rat has to enter the opposite arm it had visited in previous trial in order to get rewarded. In each session of ten trials, the number of errors committed (i.e., entry into the non-rewarded arm) was recorded. The rats were considered to have performed the task successfully only when they made eight correct choices out of ten-trials/session (criterion of acquisition). The number of correct choices per session and the time taken to reach the goal area (latency) were recorded. A rat has been considered to have entered a particular arm when it enters that arm with all its four limbs.

**Learning criterion**

To assess the acquisition of rewarded alternation task the criterion was set in such a way that the rat has to perform 8 correct alternations (i.e. 80 % correct responses) out of 10 trials. When the rat exhibits 80 % correct responses, it was considered to have learnt the task and the number of session to reach this criterion is termed sessions to criterion.

**Memory**

Two days following the last learning session, memory retention test was carried out. Rats were given a single session of 30 trials and the number of errors committed by each rat was recorded. After the T-maze experiments were over, the rats in all the groups were sacrificed under light ether anesthesia and the hippocampus region was separated out for histological and biochemical studies.

**Histopathological Assessments**

The excised hippocampus from experimental and control group rats were fixed in Bouin’s fluid (saturated picric acid: formalin: glacial acetic acid mixed in the ratio of 15: 5: 1) for 24 h. The next day, fixed tissue samples were washed with distilled water (5-6 times) and upgraded through a series of alcohol grades (70 %, 80 %, 90 % and absolute alcohol for 15 minutes each). The tissues were then placed in a mixture of absolute alcohol and xylene (1:1) for 15 minutes and cleared in xylene for 30 minutes. Thereafter, they were placed in a mixture of xylene and paraffin wax (1:1) for 15 minutes and then in paraffin wax for 15 minutes each (3 changes of paraffin wax). ‘L’ blocks smeared with glycerin were placed on a glass plate where the melted paraffin wax was poured and the tissues were finally embedded in paraffin wax. The paraffin blocks containing the tissues were then serially sectioned at 5 µm and placed on a clean glass slide pre-smeared with egg albumin white and processed further for the haematoxylin-eosin staining as follows: The slides containing the serial sections of tissue were cleared in xylene for 30 minutes and then downgraded through series of alcohol (absolute alcohol, 90 %, 80 %, 70 % for 5 - 10 minutes each). The slides were then washed in distilled water for 5 – 10 minutes and thereafter stained in haematoxylin stain for 45 minutes. The slides were then dipped in distilled water, and then placed in 70 % and 80 % alcohol for 5 – 10 minutes each. The slides were then counter stained with eosin for 30 seconds, then dipped in 90 % alcohol and then placed in absolute alcohol for 1 minute. The slides were then cleared in xylene for 5 – 10 minutes and the sections were then mounted in DPX.

**Biochemical Assays**

Tissue samples (hippocampal) were homogenized in requisite buffer for the assessment of oxidative stress indices, such as CAT, SOD, GST, GPx and GSH activity/ levels and the adopted procedures are shown below:

**Superoxide Dismutase (SOD, EC 1.15.1.1)**

SOD activity was assayed by measuring the inhibition of epinephrine auto-oxidation as described by Misra and Fridovich. The absorbance was recorded at 480 nm for 60 s. Results are expressed as units/mg protein.

**Catalase (CAT, EC 1.11.1.6)**

CAT activity was measured as described by Aebi. The rate constant of hydrogen peroxide (H2O2) decomposition was monitored by measuring the decrease in absorbance at 240 nm for 60s. Results are expressed as nmol of H2O2 consumed/min/mg protein.
Glutathione Peroxidase Activity (GPx, EC 1.11.1.9)
GPx activity was estimated by measuring the oxidation of DTNB as described by Rotruck et al. [1973] 11 and change in absorbance was measured at 420 nm. An enzyme unit represents a decrease in GSH concentration of 0.01 log unit/min, after subtraction of Non-enzyme rate. Results are presented as μmol of GSH consumed/min/mg protein.

Glutathione-S-transferase (GST, EC 2.5.1.18)
GST activity was estimated by the method of Habig et al. 12 by following the increase in absorbance at 340 nm using 1-chloro-2, 4-dinitrobenzene (CDNB) as substrate. The assay was conducted by monitoring the appearance of the conjugated complex of CDNB and GSH, viz., 2, 4-dinitrophenyl glutathione at 340 nm. Results are presented as nmol of 2, 4-dinitrophenyl glutathione formed/min/mg protein.

Reduced Glutathione (GSH)
Reduced glutathione content was determined using 5, 5-dithio-bis (2-nitrobenzoic acid) (DTNB) as fluorescent reagent according to the method of Ellman 13. GSH levels were monitored at 412 nm. Results are expressed as mg of GSH/g wet weight of tissue.

Protein Assay
Protein content was estimated by the method of Lowry et al. 14, using bovine serum albumen (BSA) as a standard.

Statistical Analysis
The results are expressed as Mean ± S.E. Values in parenthesis indicate percentage change, ‘+’ sign indicate increase, ‘-’ sign indicate decrease over control. Data were analyzed by employing Student’s-t test and values of p < 0.05 were considered statistically significant.

RESULTS
Induction of diabetes was evident in alloxan treated rats. Rats, which were able to achieve the required diabetic state (FBS more than 200 mg %) after alloxan administration were only included in the study. Six animals in the diabetic group died during the experimentation due to severe hyperglycaemia. Decreased food consumption (Figure 1A) was evident in rats upon long duration diabetes onset (2, 3 and 4-months), while increased fluid (water) intake was observed in all groups of diabetic rats studied (Figure 1B) Though the body weight did not differ in experimental rats initially, a drastic decrease in body weight was found in long duration diabetic onset groups (Figure 1C) and no changes were noticed with regard to the weight of hippocampus in either of the groups studied (OSI).

Behavioural Assessments
Acquisition
Changes observed in acquisition ability in rats as a consequence of alloxan induced diabetes are shown in Figure (2A). Results reveal that experimental rats acquired the task or reached the criterion in 5-6 sessions, which was statistically significant (P < 0.05), comparatively control rats took less sessions (3) to reach the criteria. Similarly long duration diabetic group took more sessions to reach the criteria when compared to short duration diabetes onset group.

Performance
The mean number of correct choices per 10-trials per session was recorded for 7-sessions (7-days) in all groups to measure the performance. Results showed that the long duration diabetes onset rats committed more errors (number) compared to short duration (Figure 2A).

Retention test
Memory was assessed in terms of the number of errors committed during a single session of 30-trials. Results showed in Figure (2 B) reveal significant reduction (P < 0.05) in memory in rats having long duration diabetes when compared to control group.

Latency
Changes observed in latency / the time taken by rats to reach the goal area from the start box (in seconds) as a consequence of long duration diabetes onset is shown in (Figure 2 C). Rats of 3 and 4-month diabetic onset took more time to reach the goal area when compared to short duration diabetic group.

Oxidative Stress Indices
A 10-fold increase in the activity levels of CAT (P < 0.05) was in 2-month onset diabetic rats while the quantum of increase was not on par in other groups (Figure 3 A). Likewise the activity levels of SOD was found increased in long duration diabetic rats (2-4 month duration) while 1-month onset of diabetes showed a negligible decrease in its activity levels (3 B). A similar trend with respect to the activity levels of GST (P < 0.05) and GPx (P < 0.05) was observed in long duration diabetic animals. Although both enzymes significantly increased in experimental rats, a higher fold increases in the level of GPx and GST was evident in 2-month onset diabetic group (Figure 3 C-D). On the contrary a significant decrease in the level of GSH (P < 0.05) was observed in long duration diabetic rats while 3-month onset diabetic group showed an increase in GSH levels (3 E).

Histopathology
Figure (4A) depicts the normal morphology of the hippocampus in control rats while presence of degenerating neurons, cytoplasmic eosinophilia, dark cells, condensed nucleus and vacuolization were observed in long duration diabetic rats (Figure 4B-4E). The atrophic changes were most striking in CA-1 and CA-3 hippocampal layers. The severity of degeneration was more pronounced in rats of 3 and 4-months duration than early onset diabetes.

DISCUSSION
In this study the diabetogenic agent, alloxan being a toxic glucose analogue, selectively destroyed the insulin-producing β-cells of the pancreas, causing an insulin-dependent diabetes mellitus with characteristics similar to type-1 diabetes in humans. Alloxan, in the presence of intracellular thiols, generates reactive oxygen species (ROS) in a cyclic reaction with its reduction product, dialuric acid thereby the β-cell toxic action of alloxan is initiated by free radicals formed in this redox reaction 15. Since pancreatic β-cell death underlies the pathogenesis of diabetes mellitus and excessively produced free radicals have been implicated in both β-cell destruction and enhancing the severity in long duration diabetes 16.
Fig 1: Diabetes and its durational effect on food and water intake in rats - A: Changes in food intake; B: Changes in water intake; C: Changes in body weight; D: changes in OSi (hippocampus).

Fig 2: Diabetes and its durational effect on the behavioral indices in rats - A: Changes in acquisition (Number of sessions taken to reach the goal area); B: Changes in retention (No. of errors committed during retention); C: Changes in Latency Period (time taken to reach the goal area).
Fig. 3: Diabetes and its durational effect on oxidative stress indices in hippocampus of rats-
A: Changes in activity levels of Catalase
B: Changes in activity levels of SOD
C: Changes in activity levels of GPx
D: Changes in activity levels of GST
E: Changes in activity levels of GSH.
The two primary mechanisms by which hyperglycaemia may promote the generation of ROS are the activation of the polyol pathway and increased glucose auto-oxidation. Enhanced ROS concentrations resulting from these mechanisms can cause general damage to proteins through cross-linking, fragmentation, and lipid oxidation. Another adverse effect of hyperglycaemia is the non-enzymatic glycosylation of proteins. As a function of time and glucose concentration, protein-amino groups react with glucose eventually form advanced glycosylation end products. Oxidative stress associated with hyperglycaemia may lead to a reduced number of glucose transporters and impairment of insulin secretion and oxidative stress can even have adverse effects on insulin secretion. In this study the activity levels of CAT, SOD, GST, GPx and GSH levels altered considerably and indicate a greater impact of free radical damage. The excessively produced free radicals in long duration diabetes onset may exert more and more cytotoxic effects by peroxidation of membrane phospholipids leading to change in permeability and loss of membrane integrity in functional hippocampal sub regions. Learning and memory has been associated with hippocampal activity. Literature showing studies on children with onset of diabetes before the age of six have revealed a high incidence of mesial temporal lobe sclerosis, which is shown to affect cognitive functions. Field excitatory postsynaptic potentials recorded from hippocampal slices of diabetic subjects also showed defects in hippocampal synaptic plasticity linking to difficulties in learning and memory. Behavioural assessments made in this study showed the evidence of impaired learning in diabetic animals, compared with that of normal rats. This effect has worsened with increasing duration of hyperglycaemia. Long duration (3 and 4-months) diabetes onset rats performed poorly and took longer time to reach the goal area when compared to 2 and 1-month duration diabetic rats however, 2 and 1-month duration diabetes onset rats also showed poor performance to lesser extent and considerable variation in time to reach the goal area. Long duration diabetic state impaired the acquisition, performance, latency time and retention to a greater extent. The rats put-up with 4-months of diabetes shown to be approximately equivalent to 10 years of human life revealed more cognitive deficits compared to their 1 and 2-months counterparts. Many studies link cognitive deficits in diabetes to the probable mechanisms such as hyperglycaemia induced end organ neuronal damage, dyslipidemia, amyloidopathy and tauopathy. In the present study, diabetic rats have not received any interventions like insulin, which helps to prevent neuronal damage. Hence untreated hyperglycaemia for a longer duration could be one of the causes for diabetic encephalopathy. Hippocampus is the neural structure comprising three main areas, the Cornu Ammonis (CA) regions, CA-1 and CA-3 and dentate gyrus (DG). Hippocampal CA-3 region is important in acquisition of novel information, while CA-1 is important in long term consolidation of acquired information. It plays a central role in learning and memory by performing multiple mnemonic processes such as formation of short term working memory, consolidation of information into long term storage, retrieval of information. Different hippocampal sub regions are shown to be involved in different mnemonic processes. Since hyperglycaemia is known to be toxic, higher levels of glucose found to cause vulnerable changes in long duration diabetes. The atrophic dendritic changes observed in this study as a consequence of long duration diabetic onset, and
the degenerative changes were most striking in the cells of CA-1 and CA-3 hippocampal region (Figure 4). Since hippocampus is the key tissue related to learning and memory, it is plausible that there is a direct relationship between IQ and histopathological changes of brain, thereby prolonged diabetic complications impaired the acquisition, performance, latency time and retention. Suppression of the four aforesaid parameters is indicative of decrease in the basal excitability of pyramidal neurons. Therefore, the results of the present study indicate that long term diabetic complications could cause a decrease in the excitability of both CA-1 and CA-3 neurons. In summary, diabetic encephalopathy occurs due to direct metabolic perturbations caused by hyperinsulinaemia resulted hyperglycaemia. The findings of this study strongly advocate that the learning ability and memory have a direct relation to the duration of the diabetes and the underlying mechanisms appears to be due to degenerative changes in CA-1 and CA-3 hippocampal layers. These changes may form a neural basis for impaired learning and memory, abnormal behavioural patterns, and disturbed overall body physiology. Chronic hyperglycaemia and the consequent occurrence of diabetes complications as well as recurrent episodes of severe hypoglycaemia have been shown to increase the magnitude of cognitive dysfunction in rats with type-1 diabetes. The clinical inference of the study highlights the importance of early diagnosis and treatment of juvenile diabetes induced cognitive deficits among children by controlling hyperglycaemia.

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