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Correlation between prolidase activity and spatial memory functions in streptozotocin-induced diabetic rats

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In the present study, we investigated the association of prolidase activity in streptozotocin (STZ) induced diabetic rat model with hippocampus-dependent spatial memory functions in chronic hyperglycemic conditions. Towards this, a single dose of STZ (30 mg/kg, i.p.) was administered to 4-month-old female Sprague Dawley rats and hyperglycemia was confirmed in all the STZ treated rats. To assess the spatial memory, at the time intervals of 9 days, 30 days and 90 days post STZ administration, the animals were subjected to Barnes maze task. Prolidase activity and glucose concentration in the serum were measured at these time points. With prolonged duration of hyperglycemia, severe impairment in spatial memory functions was seen in STZ induced diabetic rats which was correlatable with reduction in prolidase levels (P <0.05), thus suggesting that alterations in synaptic integrity due to prolidase mediated extracellular matrix remodeling as the possible mechanism of memory defects observed during chronic diabetes.

Keywords: Diabetes, Prolidase, Spatial memory, Streptozotocin

Diabetes mellitus, a metabolic disorder which causes impaired insulin signaling and glucose metabolism affects the synaptic plasticity in brain, particularly in hippocampus, leading to memory impairments[1]. Chronic hyperglycemia, through non-enzymatic glycation of extracellular matrix (ECM) proteins, structurally impairs the attachment of neurons to ECM[2] and functionally alters the synaptic plasticity[3]. Further, structural and biochemical alterations and rigidity in ECM have been linked to cognitive dysfunction in experimental mouse models[4]. Matrix metalloproteases (MMPs), the proteolytic enzymes responsible for ECM remodeling and repair are modulated under such pathological conditions. The peptides generated by actions of MMPs, especially dipeptide containing C- terminal proline or hydroxy proline are further acted upon by yet another iminopeptidase, prolidase which is involved in ECM remodeling and spatial organization of molecules and cells[5]. Studies with *dal* mutant mice deficient for prolidase enzyme showed morphological defects in brain as evidenced by thinner collagen fibrils, loss of pial basement membrane integrity and disrupted cytoarchitecture as a result of altered ECM remodeling[6,7].

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Prolidase is an important enzyme involved in the final step of degradation of collagen and recycling of proline. Another vital role of prolidase in CNS is the degradation of active neuropeptides, growth factors and cytokines that are rich in proline, and thus activation or inhibition of prolidase can cause significant physiological changes[8]. However, direct relation between diabetes and prolidase in hippocampus-dependent spatial memory has not been established yet.

In the current study, streptozotocin (STZ) was used to induce diabetes in adult female Sprague-Dawley (SD) rats and considered as a suitable model for hippocampus-dependent spatial memory assessments owing to the fact that systemic perturbations in glucose level cause impaired insulin signaling and increased GSK3β activity resulting in exacerbated Aβ expression[9] and tau hyperphosphorylation in the hippocampus, both of which lead to deficits in learning and memory[10-12]. With this background, an attempt has been made to elucidate the relationship of hippocampus-dependent spatial memory with prolidase, an MMP involved in ECM remodeling associated with neurodegeneration under chronic diabetic conditions. Towards this, serum prolidase activity was measured using gly-pro as the substrate. Spatial memory functions were assessed by Barnes maze task. The results suggest a positive correlation between prolidase activity and spatial memory.

Materials and Methods

Animals

Four-month-old Sprague Dawley female rats weighing 200-240 g procured from the Institute's Central Animal Research Facility were housed in standard laboratory conditions. They were maintained under 12 h light/dark cycle and had free access to food and water *ad libitum*. All the experiments involved in the study were approved by the Institutional Animal Ethics Committee (IAEC number AEC/69/447/NC).

Chemicals

Streptozotocin, Gly-pro,porcine kidney prolidase, glucose assay kit,manganese chloride and Triton-X 100 were purchased from Sigma-Aldrich, Bangalore. L-Proline, reduced L-glutathione, ninhydrin were purchased from Spectrochem Chemicals, Bangalore. All other chemicals and reagents used were of analytical grade and obtained locally.

Experimental design

The study consisted of four groups of rats (n=5 per group) as follows. Group I, Control, untreated rats; Group II-IV- Rats treated with STZ and memory recall in Barnes maze conducted for 9, 30 and 90 days post STZ administration, respectively. After the Barnes maze experiment, the blood samples were collected by cardiac puncture and the serum was separated by centrifugation at room temperature for 15 min at 5000×g and stored in aliquots at −20°C until utilization for biochemical analysis (Fig. 1).

Induction of diabetes

A single dose of streptozotocin (30 mg/kg body wt.) dissolved in 250 µL of 0.1 M citrate buffer, pH 4.5 was injected via i.p. route to experimental rats (Gr II-IV) and after three days of administration of STZ, for testing, blood samples were collected after overnight fasting and the blood glucose concentration was measured using glucose oxidase/peroxidase method using the commercially available kit (Sigma Aldrich, Bangalore). It was confirmed that all the STZ treated animals attained diabetic state after 72 h of STZ injection and remained hyperglycemic till the experimental end point.

Barnes maze test

The animals were subjected to behavioral test using Barnes maze task at definite time intervals of 9, 30 and 90 days post STZ administration to evaluate the spatial learning and memory[13]. The maze consisted of an open circular brightly lit platform with 12 holes with only one hole leading to a dark escape box. The positioning of the box remained constant throughout the entire task. The behavioural test consisted of two habituation trials on the first day and nine acquisition trials (three trials/day) over a period of next three days. After a gap of four days, retention of memory was evaluated by three trials (one day). The platform and escape box were cleaned after every trial with 70% ethanol. The number of incorrect holes explored (nose pokes in each hole), counted as errors, and the time taken (latency) to find the escape box by each group during the acquisition phase and the day of memory retention was recorded and compared with the performance of control.

Prolidase activity assay

Prolidase activity in serum was determined using colorimetric method as described by Myara *et al*.[14] and

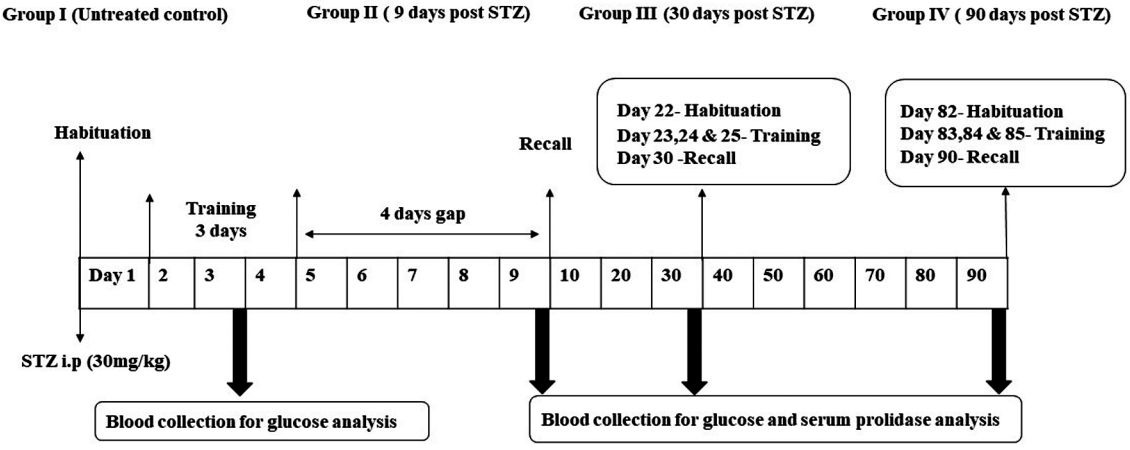

Fig. 1 — Experimental design. The nomenclature of the experimental groups of rats, time lines of administration of streptozotocin (STZ); habituation, training and recall phases of Barnes maze task; and blood sample collection for estimation of glucose levels and prolidase activity in the serum. (n=5 in each group)

the proline levels liberated enzymatically from an exogenous glycyl-L-proline (Gly-Pro) substrate was quantitated using Chinard's reagent[15]. Briefly, 10 µL of serum sample was added to 500 µL of assay buffer [50 mM Tris–HCl, pH 8.0, containing 40 mM $MnCl_2$ and 0.4 mM GSH] followed by incubation for 30 min at 40°C to activate the enzyme. Subsequently, 500 µL of substrate mix containing 40 mM Gly-Pro in 50 mM Tris–HCl, pH 8.0 and 15 mM $MnCl_2$ was added to the preincubated serum and the reaction continued at 40°C for 15 min. The activity measured with 1 U of commercially available porcine kidney prolidase served as reference. The reaction was then stopped by adding 50 µL of 0.45 M trichloroacetic acid. The samples were then centrifuged for 15 min at 5,000×g, and 0.25 mL of supernatant was used for proline estimation. For this, to 0.25 mL of the supernatant, 0.5 mL of Chinard's reagent (2.5% ninhydrin dissolved by heating at 70°C in a mix of 60 parts of glacial acetic acid and 40 parts of orthophosphoric acid) and 0.5 mL of glacial acetic acid were added and the mixture was incubated for 10min at 90°C. The amount of proline released was determined by measuring the absorbance at 515 nm using UV-Vis spectrophotometer. Enzyme activity was reported as U/L.

Statistical analysis

All data analysis was carried out using Graph pad prism software (version 8.0.1). Data are presented as mean ± S.E.M. P value less than 0.05 was considered statistically significant. Barnes maze data for learning was analyzed using two-way ANOVA. Student's unpaired *t*-test was used to compare each group with control, and the correlation between the parameters were analyzed by Pearson's correlation.

Results

In this study, the average initial weight of rats was 220 ± 21 g. At the end of experiments, the control group gained weight with progression of time (279 ± 25 g) whereas the STZ treated diabetes group weighed lesser (246 ± 19 g) to that of the control group. It was confirmed that all the STZ treated rats which had attained diabetic state after 72 h of injection (glucose concentration > 100 mg/dL) remained hyperglycemic till the experimental end point (Fig. 2A).

Both the control and STZ-treated rats with acute to chronic diabetes was evaluated by the Barnes maze test for hippocampus dependent spatial memory acquisition and retention at various time points. The aversion towards bright light motivates the animals to seek the dark escape hole hidden beneath the maze platform. Neither of the animal groups showed any significant difference in the latency or errors made during the learning phase. However, during memory recall, all the experimental groups made a greater number of errors in locating the escape hole when compared to controls, with Gr III (30 days) and Gr. IV (90 days) showing many more errors which were statistically significant indicating impairment in spatial memory (Fig. 2B) with chronicity of hyperglycemic state. The activity levels of prolidase enzyme in the serum samples gradually decreased with chronicity of diabetes in STZ treated rats when compared with control. Even though the prolidase activity in rats was reduced in Group II rats

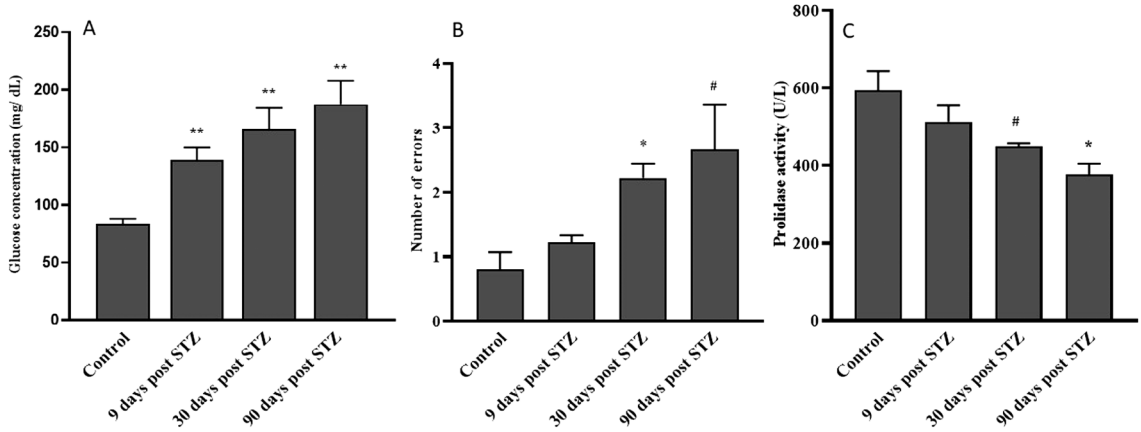

Fig. 2 — Biochemical analyses and behavioural assessments of control and STZ treated rats. (A) Fasting blood glucose levels were significantly elevated in upon STZ treatment over the control animals. P <0.01 (**); (B) During memory recall in Barnes maze task, ≥30 days post STZ Groups of rats committed greater number of errorsin comparison to the untreated control rats. P <0 .05 (*), P<0.001 (#); and(C) Serum prolidase activity gradually decreased with increase in the duration of hyperglycemic state in STZ treated rats. P <0 .05 (*), P <0.0744 (#)

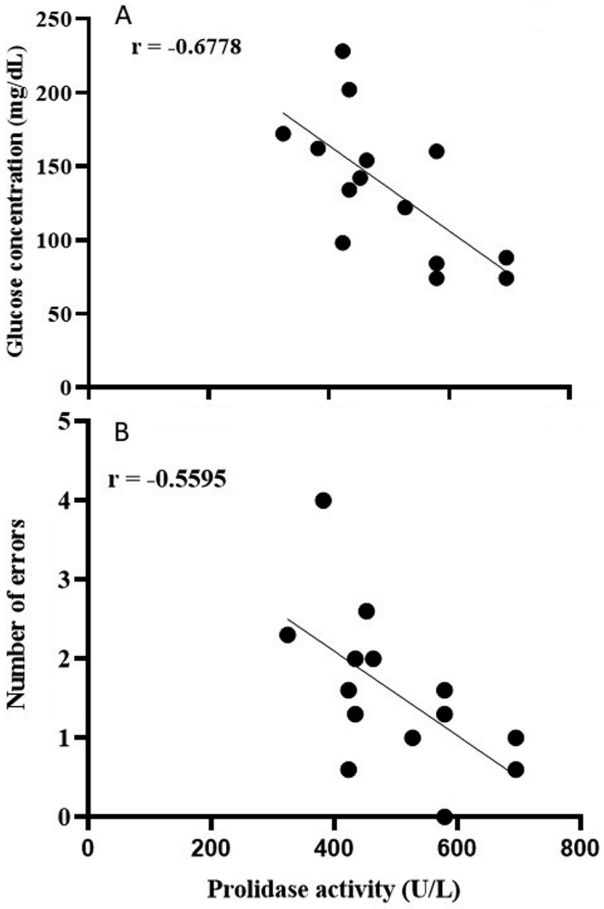

Fig. 3 — Correlation of prolidase activity with serum glucose levels and memory deficits in Barnes maze task. (A) Prolidase activity was negatively correlated with glucose concentration when analysed using Pearson's correlation coefficient.(r=-0.6778, $^{**}P$ <0.01); and (B) Prolidase activity was negatively correlated with memory deficits when analysed using Pearson's correlation coefficient (r=-0.5995, $^{*}P$ <0.05)

(9 days post STZ injection), it was statistically not significant. A trend in the statistically significant difference in prolidase activity between control and Group III (30 days post STZ) (p=0.0744) was noted. More importantly, in Group IV rats (90 days post STZ administration), a significant reduction in prolidase activity was observed (Fig. 2C). With increased glucose concentration in post STZ treated groups, a concomitant decrease in prolidase activity was observed suggesting a negative correlation between these parameters (Fig. 3A). With respect to memory function, it was observed that prolidase activity reduced with increase in the number of errors made in Barnes maze by the STZ treated rats. This indicates a negative correlation of prolidase with memory deficits and/or positive correlation with memory function (Fig. 3B).

Discussion

Clinical and epidemiological studies have confirmed that impaired metabolic parameter associated with diabetes like hyperglycemia, perturbed function of insulin, and IGF-1 signalling correlated positively with development of cognitive deficits[16,17]. Intraperitoneal STZ injection impairs brain insulin signalling pathway and GSK 3β activation in hippocampus by decreasing the inhibitory phosphorylation at serine 9 site[18]. Increased GSK 3β activity results in increase in tau phosphorylation and accumulation in hippocampus contributing to neuronal dysfunction and cognitive impairment[19]. During the state of hyperglycemia, reduction in protein synthesis such as SNAP-25[20] coupled with glycation of ECM proteins such as collagen and the concentration of selective adenosine receptors (A_2ARs) in the hippocampus[21] may play a prominent role in controlling the synaptic damage[22,23].

In the present study, poor performance in Branes maze exhibited by the STZ- treated rats is attributed to decreased synaptic strength as a result of aberrant ECM modification due to reduced prolidase activity under chronic hyperglycemic conditions. Additional functions of prolidase including degradation of endogenous dipeptides for proline regeneration and recycling may also be affected during chronic diabetes. It has recently been reported that decreased prolidase activity impairs the effective recycling of proline containing dipeptides and indirectly results in accumulation of proline-rich proteins[24]. This results in accumulation of these dipeptides as aggregosomes, suggested to interfere with synaptic connectivity. Subsequently, these proline-rich proteins induce apoptotic signal and neuronal cell loss. On similar lines, in the present study, the compromise in spatial memory in STZ induced diabetic rats could be due to alterations in ECM modulating enzymes, in particular, the prolidase activity. The reduction in prolidase activity observed in the present study is supported by the evidence available in the literature regarding diabetes induced impairment in IGF-1 signaling with lowered nitric oxide levels as the subsequent downstream events[25]. A recent study has further suggested that diabetes induces reduction in IGF-1 level in STZ treated rats[26]. It is pertinent to mention at this juncture that prolidase is primarily regulated by IGF-1 and NO levels[27,28].

Conclusion

In the present study, the results of prolidase activity strongly correlated inversely with glucose levels in diabetic rats. More importantly, the enzyme activity

negatively correlated with memory deficits and positively correlated with memory functions in the present study. These results imply that, in principle, prolidase activity can serve as an indicator of spatial memory deficits often noted in chronic diabetes. However, further studies are warranted to elucidate the mechanism of prolidase mediated progression/ recovery from memory deficits associated with diabetes.

Conflict of interest

Authors report no conflict of interest.

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