Original Article

Effects of Duration of Diabetes on Behavioural and Cognitive Parameters in Streptozotocin-Induced Juvenile Diabetic Rats

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Abstract

Background: **Diabetic encephalopathy is a recently recognised complication of early-onset type 1 diabetes in children. The abnormalities underlying diabetic encephalopathy are complex and poorly understood, and the impact of disease duration on behavioural and cognitive parameters also remains unclear. Hence, the present study was conducted to determine the effects of different durations of hyperglycaemia on behavioural and cognitive parameters in young streptozotocin-induced diabetic rats.**

Methods: **Diabetes was induced in young, weaned, age-matched rat pups by streptozotocin injection (50 mg/kg body weight, intraperitoneally). Diabetic status was confirmed on post-natal day 30. The rats were tested in the elevated plus maze 10 and 20 days after diabetes induction.**

Results: **Diabetic rats had significantly impaired behavioural and cognitive functions compared with age-matched controls. Increased anxiety levels and cognitive deficits were observed in rats that had been diabetic for 20 days compared with their 10-day counterparts.**

Conclusion: **It is essential to diagnose and treat early-onset type 1 diabetes in young children to prevent irreversible cognitive dysfunction.**

Keywords: *anxiety, cognition, juvenile-onset diabetes mellitus, learning, maze learning, memory, rats*

Introduction

Behavioural and cognitive changes associated with type 1 diabetes mellitus (T1DM) have recently gained attention. Concerns about the deleterious effects of T1DM on the central nervous system have grown with the increasing incidence of T1DM in children (1). Many studies have clearly shown cognitive and behavioural changes in type 1 diabetic rats and humans, which are evident in elevated levels of anxiety, depression, and slowing of mental speed and flexibility (2–5).

Diabetes-induced behavioural and cognitive changes are related to several factors. Both diabetic complications and reduced central serotonin (5-hydroxytryptamine, 5-HT) synthesis and metabolism are thought to underlie behavioural and cognitive dysfunctions in patients with T1DM (6,7). It has become evident that insulin and C-peptide deficiencies, including perturbations of their signalling cascades, lead to cerebral dysmetabolism and interference with the regulation of neurotropic factors and their receptors. Ultimately, this cascade of events leads to neuronal loss, causing profound deficits in behavioural and cognitive functions (8). However, the specific mechanisms underlying these changes and whether they relate to the duration of hyperglycaemia are unknown.

Although the magnitude of most of these cognitive decrements is relatively modest, even moderate behavioural and cognitive changes can potentially hamper the day-to-day activities of a diabetic child. These cognitive decrements may present problems in more demanding situations, and critically, can have a negative impact on the quality of life.

Patients with an onset of diabetes before the age of 5 years may be more sensitive to diabetic complications and diabetic effects related to encephalopathy. Many researchers have shown that there is a relationship between neuropsychological changes and early-onset T1DM (3,9,10). The contributions of several

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disease variables, such as diabetic duration, level of glycaemic control and the developmental trajectory of neuropsychological impairments, remain unresolved. Hence, the present study was conducted to evaluate the effects of different diabetic durations on various behavioural and cognitive parameters using the elevated plus maze (EPM) in streptozotocin (STZ)-induced diabetic rat pups.

Materials and Methods

Inbred male and female Wistar rats, 25 days old with weights of 45–50 g, were selected for the study. Experiments were approved by the Institutional Animal Ethical Committee (627/02/a/CPCSEA dated 17 July 2008). The rats were maintained on a 12:12-hour light:dark cycle under controlled temperatures (25 °C, SD 3) and had ad libitum access to food (Amrut feeds, standard rat pellets) and water. All experiments were performed between 08:00 and 16:00 hour. Rats were randomly divided into the following groups of 6 rats each:

- Group 1 (N-10): Control for 10 days
- Group 2 (D-10): Diabetic for 10 days
- Group 3 (N-20): Control for 20 days
- Group 4 (D-20): Diabetic for 20 days

Rats in the diabetic groups received an intraperitoneal injection of STZ (50 mg/kg) on post-natal day 25. Four days later, blood was collected from the tail vein following an overnight fast (11–13). Fasting blood sugar (FBS) was measured with a standard glucometer (Optium, Germany), and the day that diabetes was confirmed was considered to be diabetic day 1. Rats with FBS lower than 200 mg/dL were excluded from the study. Eleven days after diabetes confirmation, rats in the D-10 group were assessed for cognitive and behavioural parameters in the EPM. Similarly, rats in the D-20 group were also assessed with the same measures 21 days after diabetes confirmation. The details of the EPM test are explained below.

The EPM is widely used for rodent neuropsychological assays, such as anxiety behaviour as well as learning and memory tests, and valid results can be obtained in a short, 5-minute testing period. The maze consists of 4 arms (2 open arms without walls, OAs, and 2 arms enclosed by 30-cm-high walls, EAs), 50 cm long and 10 cm wide, that are attached to a central platform (5 × 5 cm) at right angles. The apparatus is elevated to a height of 50 cm above the floor and is kept in a brightly lit room.

Anxiety protocol

The rats were placed on the central platform with their heads oriented towards an OA. The frequency of entries into the OAs and EAs were scored and time spent in the OAs was recorded for 5 minutes. The number of entries into the OAs of the maze and the time spent in those arms are the measures of anxiety, and decreases in these measures indicate an anxiogenic effect. The number of EA entries serves as the measure of locomotor activity in this test. An arm entry is defined as all 4 paws entering an arm, and an arm exit is defined as 2 paws leaving an arm. During this period, ethological parameters such as number of rears, grooming, and boli of excreta were also counted.

Learning protocol

A line was drawn to divide the EA into 2 equal parts. On days 1 and 2, we measured the time it took for each rat to cross the line in the EA (transfer latency). The rat was initially placed at the end of an OA and allowed to explore for 90 seconds. The rat was required to have its body and 4 paws cross the line in the EA; if the rat did not cross the line after the time limit, it was manually placed beyond the line and transfer latency was recorded as 90 seconds. After crossing the line, the rat was allowed to spend 30 seconds exploring the apparatus. Learning was defined as reduced transfer latency on day 2 compared with day 1. Over the test period, normal rats typically cross the line in the EA more quickly on day 2 than on day 1 (14–17).

Statistical analysis

The results are expressed as means and standard deviations (SD). The between-group comparisons of FBS were made with unpaired Student's *t* tests. For behavioural measures from the EPM, the between-group comparisons were made with Mann–Whitney U non-parametric tests. Differences were considered significant at $P < 0.05$.

Results

We randomly assigned 72 rats to different experimental groups. There were 6 rats in each of the control groups (N-10 and N-20). The remaining 50 rats were given STZ injections to induce diabetes. Out of the 50 rats, 22 died and 28 became diabetic; 15 of the diabetic rats achieved the required diabetic state (FBS greater than 200 mg/dL) and were included in the study (D-10 and D-20). During the study period, 3 of

the diabetic rats died. Data collected from the 4 groups of rats, control (N-10 and N-20) and diabetic (D-10 and D-20), are summarised below.

FBS was measured on post-natal days 30 and 60, and the results are shown in Table 1. A statistically significant difference ($P < 0.001$) observed in FBS values on post-natal day 30 between diabetic rats and their respective age-matched controls. The severity of diabetes increased over time, showing significant differences in FBS levels between post-natal days 30 and 60 ($P = 0.018$) in diabetic rats with a 20-day hyperglycaemia duration. The diabetic rats in the D-10 group were sacrificed immediately after the tests and processed further for neurohistological studies.

The EPM performances are shown in Table 2. Anxiety tests indicate that the diabetic rats spent less time in the OA and made fewer arm entries compared with the control rats. The statistically significant differences were observed in the number of OA entries ($P = 0.009$) and the time spent in the OA ($P = 0.006$) between D-20 rats and their age-matched controls. D-10 rats showed no showed no significant differences compared with their age-matched controls. No significant differences were observed in other behavioural (ethological) parameters, such as rearing, grooming, and number of boli excreted in both D-10 and D-20 groups compared with their age-matched controls. Furthermore, the number of EA entries did not differ between diabetic and

Table 1: The effects of diabetes duration on fasting blood sugar (FBS) level in normal control and streptozotocin-induced diabetic rats

Group	FBS (mg/dL)	
	Post-natal day 30	Post-natal day 60
Control—10 days	87.3 (3.85)	-
Diabetic—10 days	233.0 (10.27) [a]	-
Control—20 days	86.8 (3.37)	89.0 (3.95)
Diabetic—20 days	269.1 (20.41) [a]	319.3 (38.51) [a,b]

Each group consisted of 6 rats. All values are expressed as mean (SD).
[a] Significant difference ($P < 0.05$) compared with the respective normal control by unpaired Student's *t* tests.
[b] Significant difference ($P < 0.05$) compared with the post-natal day 30 by paired Student's *t* tests.

Table 2: The effects of diabetes duration on anxiety level in control and streptozotocin-induced diabetic rats in the elevated plus maze

Group	No. of entries		Time in OA (seconds)	Ethological parameters		
	EA	OA		Rearing	Grooming	No. of boli excreted
Control—10 days	4.1 (0.98)	1.83 (0.16)	13 (0.77)	6.1 (0.47)	2.6 (0.49)	1.1 (0.30)
Diabetic—10 days	3.3 (0.33)	0.83 (0.75)	6.16 (3.09)	4.8 (0.60)	3.1 (0.47)	1.8 (0.60)
Control—20 days	6.6 (0.61)	4.3 (1.23)	64.3 (20.87)	9.6 (0.88)	2.1 (0.47)	0.1 (0.16)
Diabetic —20 days	5.6 (0.92) [b]	1 (0.36) [a,b]	9.5 (3.66) [a]	8.1 (0.94) [b]	3.3 (0.33)	0.8 (0.47) [b]

Each group consisted of 6 rats. All values are expressed as mean (SD).
[a] Significant difference ($P < 0.05$) compared with the respective normal control by Mann–Whitney non-parametric tests.
[b] Significant difference ($P < 0.05$) compared with the 10-day diabetic rats by Mann–Whitney non-parametric tests.
Abbreviations: EA = enclosed arm, OA = open arm.

normal rats. Rats in the D-20 group differed significantly from D-10 group in the number of entries into EA (P = 0.002) and into OA (P = 0.004), as well the numbers of rears (P < 0.001) and boli of excreta (P = 0.009).

The transfer latency results are summarised in Table 3. On day 1, there were significant differences in the transfer latency results of D-10 (P = 0.039) and D-20 (P = 0.006) rats compared with their respective controls on day 1 in the learning paradigm. Significant differences were also observed on day 2 in D-10 (P = 0.009) and D-20 (P = 0.02) rats compared with the controls in the memory retention trials. In addition, the transfer latencies were significantly difference between D-10 and D-20 groups on both day 1 (P = 0.03) and day 2 (P = 0.013).

Discussion

STZ-induced diabetic rat is a well-established animal model of diabetes. Intra-peritoneal injection of STZ induces "chemical diabetes" in a wide variety of animal species, including rats, by selectively damaging the insulin-secreting β cells of the pancreas, as evidenced by their clinical symptoms of hyperglycaemia and hypoinsulinaemia (18).

The EPM is a widely accepted test in the study of anxiety in rodents and other animal models (17,19–24). The EPM is also sensitive enough to detect deficits in associative learning and memory in rats (14).

The EPM results revealed increased anxiety in diabetic rats compared with control rats, which was evident in the decreased number of OA entries and less time spent in the OA. However, no significant differences in the number of EA entries were observed, suggesting no gross locomotor activity changes in the diabetic rats. The ethological measures such as rears, grooming, and number of excreted boli also did not differ significantly between groups, although modest differences were observed. Significant differences were seen between rats in D-10 and D-20 groups. The increased anxiety levels associated with longer diabetic duration might have worsened cerebral dysmetabolism. Many studies have stated that anxiety in diabetic rats could be attributed to 5-HT, adenylyl cyclase type VIII, and tuberoinfundibular peptide of 39 residues deficiencies (7,8,25,26).

The EPM learning and memory measures from day-1 and day-2 trials showed that the groups of diabetic rats differed significantly, not only compared with normal controls, but also between themselves (D-10 and D-20). This clearly suggests that cognitive decline worsens with increasing duration of hyperglycaemia. The condition of rats in the D-20 group is approximately equivalent to 2 years of diabetes in a human life, and they showed increased cognitive deficits compared with their 10-day counterparts. Many studies link these diabetic cognitive deficits to hyperglycaemia-induced end-organ neuronal damage, dyslipidaemia, amyloidopathy, and tauopathy, among other causes (27–29). In the present study, diabetic rats did not receive any intervention, such as insulin, that would have prevented the neuronal damage. Hence, untreated hyperglycaemia for long durations may be one cause of diabetic encephalopathy. Other consequences of insulin deficits and perturbations include innate inflammatory responses affecting synaptogenesis and neuronal degeneration. Eventually, this cascade of events leads to more profound deficits in behavioural and cognitive functions due to extensive neuronal loss and decreased white matter density of myelinated cells. Neuroimaging data suggest white matter

Table 3: The effects of diabetes duration on learning and memory in control and STZ-induced diabetic rats in the elevated plus maze

Group	Transfer latency (seconds)	
	Day 1	Day 2
Control—10 days	63.0 (6.32)	25.0 (4.83)
Diabetic—10 days	81.0 (4.18) [a]	60.0 (9.69) [a]
Control—20 days	46.6 (10.07)	14.1 (2.28)
Diabetic—20 days	87.8 (1.51) [a, b]	72.8 (14.00) [a, b]

Each group consisted of 6 rats. All values are expressed as mean (SD).

[a] Significant difference (P < 0.05) compared with the respective normal control by Mann-Whitney non-parametric tests.

[b] Significant differences (P < 0.05) compared with the 10-day diabetic rats by Mann-Whitney non-parametric tests.

atrophy in the frontal and temporal brain regions, which could be linked to deficits in certain cognitive domains such as memory, information processing speed, executive function, attention, and motor skill speed. Morphological studies of children with diabetic onset before the age of 6 have revealed a high incidence of mesial temporal lobe sclerosis, which is not associated with a history of hypoglycaemia (8,30). Interestingly, deficits in such cognitive functions are also associated with impaired functional connectivity, which is a measure of functional interactions among brain regions (31). Field excitatory post-synaptic potentials recorded from hippocampal slices of diabetic rats show defects in the induction of hippocampal synaptic plasticity that are linked to difficulties in learning and memory (32).

The present study was designed to investigate the effects of different diabetic durations on behavioural and cognitive dysfunction in early life stages. Using an STZ-induced diabetic model, we found that, in young rats, the diabetic duration significantly contributes to learning and memory deficits, which were irreversible, and to the induction of high levels of anxiety.

Conclusion

It has recently become clear that the central nervous system is not spared from the deleterious effects of diabetes. Diabetic encephalopathy is primarily caused by the direct metabolic perturbations of hyperglycaemia, insulin deficiency, or hypoinsulinaemia. Secondary diabetic encephalopathy occurs as a result of micro- and macrovascular disorders or due to repeated episodes of hypoglycaemia induced by excess insulin (33–35). The results of this study suggest that behavioural and cognitive changes are directly related to the duration of the diabetic state; however, the underlying mechanisms remain unknown. This study highlights the clinical importance of early diagnosis and treatment of juvenile diabetes and associated neuropsychological deficits in children.

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Authors' Contributions

Conception and design, critical revision and final approval of the article: RR, SDK, SSG
Obtaining of funding, provision of study materials, collection, assembly, analysis, and interpretation of the data, statistical expertise, drafting of the article: RR

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References

1. Adeghate E, Schattner P, Dunn E. An update on the etiology and epidemiology of diabetes mellitus. *Ann N Y Acad Sci*. 2006; **1084**:1–29.
2. Ramanathan M, Jaiswal AK, Bhattacharya SK. Differential effects of diazepam on anxiety in streptozotocin induced diabetic and non-diabetic rats. *Psychopharmacology (Berl)*. 1998;**135(4)**:361–367.
3. Kuhad A, Chopra K. Curcumin attenuate diabetic encephalopathy in rats: Behavioral and biochemical evidences. *Eur J Pharmacol*. 2007;**576(1–3)**:34–42.
4. Alvarez EO, Beauquis J, Revsin Y, Banzan AM, Roig P, De Nicola AF, et al. Cognitive dysfunction and hippocampal changes in experimental type 1 diabetes. *Behav Brain Res*. 2009;**198(1)**:224–230.
5. Brands AM, Biessels GJ, De Haan EH, Kappelle LJ, Kessels RP. The effects of type 1 diabetes on cognitive performance: A meta-analysis. *Diabetes Care*. 2005;**28(3)**:726 –735.
6. Ryan CM, Williams TM. Effects of insulin-dependent diabetes on learning and memory efficiency in adults. *J Clin Exp Neuropsychol*. 1993;**15(5)**:685–700.
7. Thorre K, Chaouloff F, Sarre S, Meeusen R, Ebinger G, Michotte Y. Differential effects of restraint stress on hippocampal 5-HT metabolism and extracellular levels of 5-HT in streptozotocin-diabetic rats. *Brain Res*. 1997;**772(1–2)**:209–216.
8. Sima AA, Zhang W, Muzik O, Kreipke CW, Rafols JA, Hoffman WH. Sequential abnormalities in type 1 diabetic encephalopathy and the effects of C-Peptide. *Rev Diabet Stud*. 2009;**6(3)**:211–222.
9. Toth C, Schmidt AM, Tuor UI, Francis G, Foniok T, Brussee V, et al. Diabetes, leukoencephalopathy and rage. *Neurobiol Dis*. 2006;**23(2)**:445–461.

10. Schoenle EJ, Schoenle D, Molinari L, Largo RH. Impaired intellectual development in children with Type I diabetes: Association with HbA(1c), age at diagnosis and sex. *Diabetologia*. 2002;**45(1)**: 108–114.

11. Kulkarni SK. *Hand book of experimental pharmacology*. 3rd ed. Delhi (IN): Vallabh Prakashan; 1999.

12. Meiri N, Chelardini C, Tesco G, Galeotti N, Dahl D, Tomsic D, et al. Reversible antisense inhibition of Shaker-like Kv1.1 potassium channel expression impairs associative memory in mouse and rat. *Proc Natl Acad Sci U S A*. 1997;**94(9)**:4430–4434.

13. Walther T, Balschun D, Voigt JP, Fink H, Zuschratter W, Birchmeier C, et al. Sustained long term potentiation and anxiety in mice lacking the *Mas* protooncogene. *J Biol Chem*. 1998;**273(19)**: 11867–11873.

14. Carrie I, Clement M, de Javel D, Frances H, Bourre JM. Phospholipid supplementation reverses behavioral and biochemical alterations induced by n-3 polyunsaturated fatty acid deficiency in mice. *J Lipid Res*. 2000;**41(3)**:473–480.

15. Bures J, Buresova O, Huston JP. *Techniques and basic experiments for the study of brain and behavior*. 2nd ed. Amsterdam (NL): Elsevier Science; 1983.

16. Nayak V, Patil PA. Antidepressant activity of fosinopril, ramipril and losartan but not of lisinopril in depressive paradigms of albino rats and mice. *Indian J Exp Biol*. 2008;**46(3)**:180–184.

17. Pellow S, Chopin P, File SE, Briley M. Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *J Neurosci Methods*. 1985;**14(3)**:149–167.

18. Hofteizer B, Carpenter AM. Comparison of streptozotocin and alloxan-induced diabetes in rats, including volumetric quantization of pancreatic islet cells. *Diabetologia*. 1973;**9(3)**:178–184.

19. Rodgers RJ, Dalvi A. Anxiety, defence and the elevated plus-maze. *Neurosci Biobehav R*. 1997;**21(6)**: 801–810.

20. Cavalli J, Bertoglio LJ, Carobrez AP. Pentylenetetrazole as an unconditioned stimulus for olfactory and contextual fear conditioning in rats. *Neurobiol Learn Mem*. 2009;**92(4)**:512–518.

21. Hansen SL, Sperling BB, Sanchez C. Anticonvulsant and antiepileptogenic effects of GABA receptor ligands in pentylenetetrazole-kindled mice. *Prog Neuropsychopharmacol Biol Psychiatry*. 2004;**28(1)**:105–113.

22. De Carvalho RS, Duarte FS, de Lima TC. Involvement of GABAergic non-benzodiazepine sites in the anxiolytic-like and sedative effects of the flavonoid baicalein in mice. *Behav Brain Res*. 2011;**221(1)**: 75–82.

23. Lister RG. The use of a plus-maze to measure anxiety in the mouse. *Psychopharmacology (Berl)*. 1987;**92(2)**:180–185.

24. Setem J, Pinheiro AP, Motta VA, Morato S, Cruz AP. Ethopharmacological analysis of 5-HT ligands on the rat elevated plus-maze. *Pharmacol Biochem Behav*. 1999;**62(3)**:515–521.

25. Schaefer ML, Wong ST, Wozniak DF, Muglia LM, Liauw JA, Zhuo M, et al. Altered stress-induced anxiety in adenylyl cyclase type VIII-deficient mice. *J Neurosci*. 2000;**20(13)**:4809–4820.

26. Fegley DB, Holmes A, Riordan T, Faber CA, Weiss JR, Ma S, et al. Increased fear- and stress-related anxiety-like behavior in mice lacking tuberoinfundibular peptide of 39 residues. *Genes Brain Behav*. 2008;**7(8)**:933–942.

27. Holmes CS, Hayford JT, Gonzalez JL, Weydert JA. A survey of cognitive functioning at difference glucose levels in diabetic persons. *Diabetes Care*. 1983;**6(2)**:180– 185.

28. Kim B, Backus C, Oh S, Hayes JM, Feldman EL. Increased tau phosphorylation and cleavage in mouse models of type 1 and type 2 diabetes. *Endocrinology*. 2009;**150(12)**:5294–5301.

29. Heikkila O, Lundbom N, Timonen M, Groop PH, Heikkinen S, Makimattila S. Hyperglycaemia is associated with changes in the regional concentrations of glucose and myo-inositol within the brain. *Diabetologia*. 2009;**52(3)**:534–540.

30. Ho MS, Weller NJ, Ives FJ, Carne CL, Murray K, Vanden Driesen RI, et.al. Prevalence of structural central nervous system abnormalities in early-onset type 1 diabetes mellitus. *J Pediatr*. 2008;**153(3)**:385–390.

31. Van Duinkerkeu E, Klein M, Schoonenboom NS, Hoogma RP, Moll AC, Snoek FJ, et al. Functional brain connectivity and neurocognitive functioning in patients with longstanding type 1 diabetes with and without microvascular complications: A magnetoencephalography study. *Diabetes*. 2009;**58(10)**:2335–2343.

32. Kamal A, Biessels GJ, Gispen WH, Ramakers GM. Synaptic transmission changes in the pyramidal cells of the hippocampus in streptozotocin-induced diabetes mellitus in rats. *Brain Res*. 2006;**1073–1074**:276–80.

33. Sima AA, Kamiya H, Li ZG. Insulin, C-peptide, hyperglycemia, and central nervous system complications in diabetes. *Eur J Pharmacol*. 2004;**490(1–3)**:187–197.

34. Biessels GJ, Deary TJ, Ryan CM. Cognition and diabetes: A lifespan perspective. *Lancet Neurol*. 2008;**7(2)**:184–190.

35. Biessels GJ. Diabetic encephalopathy. In: Veves A, Malik RA, editors. *Diabetic neuropathy: Clinical management*. 2nd ed. Totowa (NJ): Humana Press Inc.; 2007. p. 187–205.