VIA MEDICA

ORIGINAL ARTICLE

The granule cell density of the dentate gyrus following administration of *Urtica dioica* extract to young diabetic rats

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Urtica dioica *L. Stinging nettle has long been known worldwide as a medicinal plant. To study the benefits of the nettle in diabetic encephalopathy, the granule cell density of the dentate gyrus of diabetic rats was studied following administration of* Urtica dioica *extract. A total of 24 male albino Wistar rats were allocated equally to normal, diabetic, preventive and treatment groups. Hyperglycaemia was induced by streptozotocin (80 mg/kg) in the animals of the diabetic and treatment groups. One week after injection of the streptozotocin the animals in the treatment group received a hydroalcoholic extract of* Urtica dioica *(100 mg/kg/day) for 4 weeks intraperitoneally. The rats of the preventive group received hydroalcoholic extract of* U. dioica *(100 mg/kg/day) IP for the first 5 days and an injection of streptozotocin (80 mg/kg) on the 6th day. After 5 weeks of study all the rats were sacrificed and coronal sections were taken from the dorsal hippocampal formation of the right cerebral hemispheres and stained with cresyl violet. The area densities of the granule cells were measured and compared in the four groups. The density was lower in the diabetic rats compared with the controls (p > 0.05). The preventive group showed lower cell density than the controls (p > 0.05). The densities in the treated rats were higher than in the diabetic rats (p > 0.05). Furthermore, the control and treated rats showed similar densities (p > 0.05). It seems that* U. dioica *extract can help compensate for granule cell loss in the diabetic rat dentate gyrus, which can ameliorate cognitive impairment in diabetes. However, preventive use of the extract showed no significant benefit. (Folia Morphol 2008; 67: 196–204)*

Key words: granule cell density, dentate gyrus, *Urtica dioica*, diabetes, rat

INTRODUCTION

Diabetes mellitus is one of the most common serious metabolic disorders in humans. Increasing evidence has shown that diabetes may be associated with learning and memory deficiency in humans [5, 6, 19]. Studies examining the effects of streptozotocin-induced diabetes on memory function in mice and rat models have also shown impairment of memory retention and retrieval as compared to non-diabetic controls [3, 4, 9, 27].

It has been suggested that the dentate gyrus, which is a part of the hippocampal formation, plays an important role in learning and memory by processing and representing spatial information on the basis of conjunctive encoding, pattern separation and encoding of spatial information in conjunction

with the CA3 region [16]. In addition, it is now well documented that new neurons are continuously being produced in the dentate gyrus of adult mammals, a process known as "adult neurogenesis" [10].

Although the exact mechanism causing the cognitive disorders of diabetes has not yet been clearly understood, some evidence suggests that changes in the granule cell and its proliferating precursors in the dentate gyrus are involved [2, 13, 24]. A granule cell decrease was found in the hilus of the dentate gyrus in diabetic mice compared with controls [2].

During the last few years consideration has been given to various strategies to prevent and treat diabetic encephalopathy, which is characterised by impaired cognitive functions and neurochemical and structural abnormalities [2, 17, 18, 24, 25, 28]. The use of plant materials as neuroprotective agents has been reported by some researchers [7, 14, 20]. The extract of these herbal materials can restore hippocampal neurogenesis and improve cognitive dysfunction in diabetic animals [20].

Urtica L., the stinging nettle, a member of the *Urticaceae* family, is a perennial herb, distinguished by its stinging hairs [15]. Among the *Urtica* species, *Urtica dioica* (*U. dioica*) has long been known worldwide as a medicinal plant. The blood sugar lowering effect of *U. dioica* as a medicinal herb is referred to in old manuscripts, such as those written by Avicenna [8]. In addition, *U. dioica* is among several species listed for their use against diabetes in folk medicine in a large pharmacological screen of European species [23].

The neuropreventive effects of *U. dioica* in the prevention and treatment of the cognitive complications of diabetes have not been clearly demonstrated. This study was conducted to study the granule cell density of the dentate gyrus following administration of *Urtica dioica* extract to diabetic rats.

MATERIAL AND METHODS

The study was performed in 2006–2007 in the Faculty of Medicine, Gorgan University of Medical Science. Approval for this study was gained from the Animal Care and Ethics Committee of the Gorgan University of Medical Sciences.

Plant material

U. dioica leaves were collected from cultivated plants, from a suburb of Gorgan, northern Iran (Golestan, Iran) and taxonomically identified by the Department of Pharmacognosy, Mazandaran University of Medical Sciences. A voucher specimen (5-77-1) was deposited in the herbarium of Mazandaran University.

Preparation of plant extract

The aerial parts of *U. dioica* were reduced to small pieces, dried in a circulating air stove and powdered in a grinder. The powdered material was then macerated using a hydroalcoholic (60°) solvent for 48 hours. The ethanol was removed by vacuum distillation and the resulting residue was filtered and concentrated at 40°C to make a jellied material. In addition to thin-layer chromatography and purity tests (for foreign matter, total ash, acid insoluble ash and water insoluble ash) for qualification analysis, another monosaccharide-linked reagent assay (spectrophotometry) was carried out to determine the concentration of polysaccharides in the *U. dioica* leaves for standardisation of the extract. The results of phytochemical analysis showed the existence of a high percentage of tannins and steroids and low levels of flavonoids, carotenoids and saponins in the leaves of *U. dioica*.

At the time of administration the prepared powder of the extract was dissolved in saline and the rats were treated with the solution.

The animals and the hyperglycaemic model

A total of 24 male albino Wistar rats aged 6–7 postnatal weeks were provided by the Iranian Pasteur Institute. The rats, weighing 158.00 ± 42.09 g, were fed on a pellet diet and tap water *ad libitum* for full acclimatisation. During 5 weeks of the experiment the animals were kept in air-conditioned animal quarters (22 ± 2°C) under a 12 hours light/dark cycle. To prevent any confounding effects on hippocampal histology, the environment was non-enriched. The rats were divided into four groups (n = 6) as follows:
— control: no injection on the first day; saline daily 2nd – 5th weeks;
— diabetic: streptozotocin (STZ) injection on the first day; saline daily 2nd – 5th weeks;
— preventive: hydroalcoholic extract of *U. dioica*, 100 mg/kg/day, IP, over the first 5 days; STZ injection in the 6th day; saline daily until the end of the experiment;
— treatment: STZ injection on the first day; hydroalcoholic extract of *U. dioica*, 100 mg/kg/day, IP, 2nd – 5th weeks.

To produce a hyperglycaemic model on the first day of the experiment, the diabetes was induced with a single IP injection of STZ (80 mg/kg) to overnight fasting rats. The STZ dose was determined by
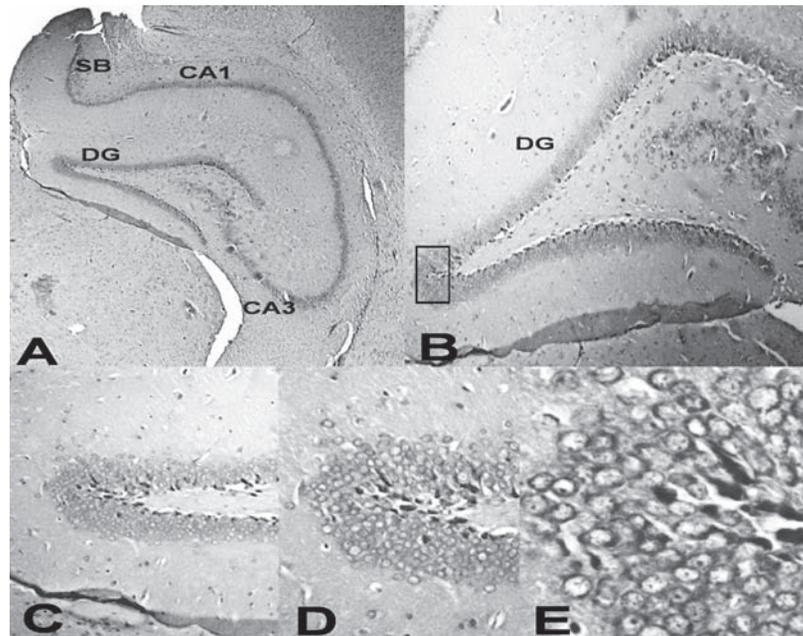
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Figure 1. Coronal sections stained with cresyl violet showing the hippocampal formation of the rat under light microscopy. **A.** The overall cytoarchitecture of hippocampal formation (40×); **B.** The zone of study was the outlined area in the dentate gyrus (100×); **C, D, E.** The zone of study with higher magnifications (200, 400, 1000×). The granule cells stained darkly and are clearly visible. SB — subiculum; DG — dentate gyrus; CA1 and CA3 — corona ammonis 1 and 3.

a prior pilot study. Streptozotocin purchased from Sigma Co. was dissolved in saline and intraperitoneally injected.

Blood glucose sampling

Blood samples for glucose measurements were taken from the tail vein of the rats. Blood glucose concentrations were measured at the beginning of the 1st, 2nd and 4th weeks using an ACCU-CHEK® Active Glucometer, Roche Diagnostics GmbH, Germany.

Glucose tolerance test

The intraperitoneal glucose tolerance test (GTT) was performed on 16 hours fasted rats using 2 g dextrose per kg body weight at the beginning of the experiment. In all groups blood was collected from the animals by tail snipping at 0, 30, 90 and 120 min after glucose load. The GTT was also performed in the 5th week.

Tissue processing

The animals of the four groups were sacrificed following ether anaesthesia. The skulls of the rats were dissected and the brains collected. After measurement of the total brain weights (i.e. hemispheres and cerebellum), the right hemispheres were separated through the corpus callosum. The hemispheres were fixed in formaldehyde 10% for 48 hours and paraffin embedded. The 7 μm coronal sections were serially collected from Bregma –3.30 mm to –6.04 mm of the hippocampal formation [21]. An interval of 20 μm was placed between each two consecutive sections. The sections were stained with cresyl violet in accordance with routine laboratory procedures.

Morphometric study

A photograph of each section was produced using an Olympus BX 51 microscope and a DP 12 digital camera under a magnification of 1000×. An area of 10000 μm^2 was selected in the hilar region of the dentate gyrus in all sections (Fig. 1). To measure the area density of the granule cells, the images were transferred to the computer. Using OLYSIA Autobioreport software, Olympus Co, the appropriate grids were superimposed on the pictures and the cells were counted manually. To perform an unbiased measurement, the individual was double-blinded and only the cells with significant granule cell characteristics were counted.

Statistical analysis

All the data were entered into and analysed by SPSS 11.5 software. To compare the means of the measured parameters in the four groups by analysis of variance (ANOVA) test, the normality of distribution was first evaluated by the Kolmogorov-
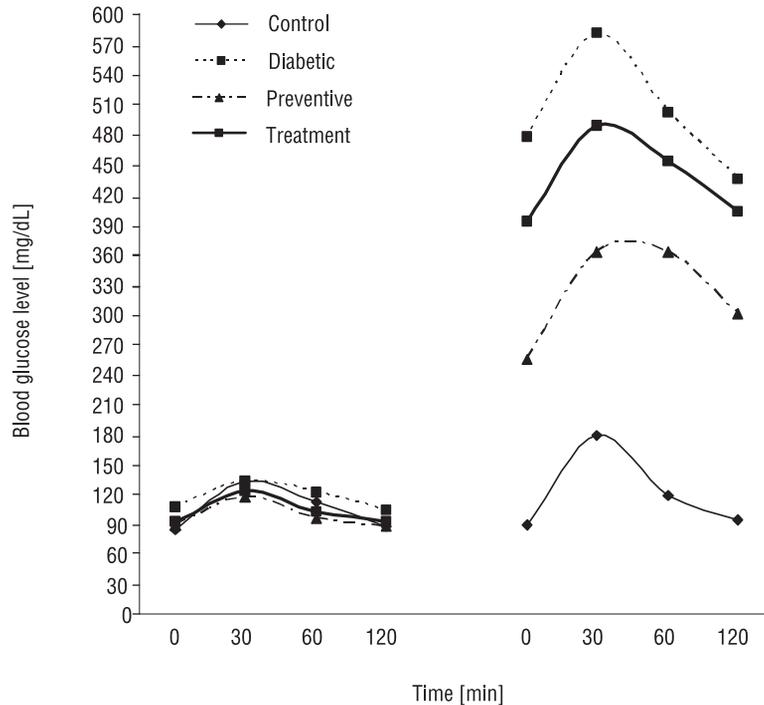
Figure 2. Glucose tolerance test of the four groups of the experiment at the beginning (left) and in the 5th week of the study (right).

-Smirnov test and, after confirmation of normality, the means were compared by the ANOVA test. For pairwise comparison of the groups, the homogeneity of variance was tested by Levene's test and, where there was homogeneity, comparison was made by a supplementary test of LSD. If there was no homogeneity, Dunnett's T3 test was used.

The correlations between granule cell density and the anterior-posterior position of the sections, mean glucose level at 5 weeks and the weight of the rat brain were individually examined by the Pearson correlation test. As the density may be affected by all the variables in a multi-factorial manner, the net effect of the variables mentioned on the density were tested with different multi-variable regression models by the "backward" method.

RESULTS

The GTT results of the four experimental groups at the beginning and in the 5th week of the study are presented in Figure 2. As shown, these were normal for all the rats at the beginning of the experiment. The GTT results at the end of the study showed a marked difference from the prior results for the rats receiving STZ, those of the diabetic, preventive and treatment groups. As shown in Figure 2, the control rats, which had not received STZ, showed a normal GTT. In contrast, the diabetic rats, which had undergone STZ-induced diabetes on the first day of study and had not received the nettle extract, showed the most profound impairment of GTT. The effects of prevention and treatment by nettle extract on the GTT results varied. Interestingly, the GTT of the preventive group showed a lower level of impairment than that of the rats treated by the nettle extract.

Table 1 shows the brain weights, body weights and brain/body weight ratios of the experiments at the end of the study. Analysis of variance among the four groups using the ANOVA test showed that there were significant differences in these values in the four groups ($p < 0.05$). Levene's test showed that variances of brain weight were homogeneous, and multiple comparisons between pairs of groups were carried out by the LSD test.

The diabetic and treatment rats showed lower brain weights than the control group ($p < 0.05$). The observed difference between the preventive and control rats was not statistically significant ($p > 0.05$). However, because of the different body weights of the animals, a comparison of the brain/body weight ratios of the experimental animals seems to be more precise. As shown in Table 1, no significant difference was seen between the ratios of the diabetic and control groups ($p > 0.05$). Similarly, the preventive group also showed

Table 1. Brain weights, body weights and brain/body weight ratios in the experimental groups at the end of study

Group	Brain weight [g]	Body weight	Brain/body weight ratio (%)
Control			
Rat no. 1	1.59	267.00	0.60
Rat no. 2	1.72	222.00	0.77
Rat no. 3	1.75	217.00	0.81
Rat no. 4	1.70	231.00	0.74
Rat no. 5	1.80	245.00	0.73
Rat no. 6	1.67	190.00	0.88
Mean ±SD	**1.70 ± 0.07[a, b,*]**	**228.67 ± 26.14[a, b, c]**	**0.75 ± 0.09[a]**
Diabetic			
Rat no. 1	1.62	173.00	0.94
Rat no. 2	1.38	137.00	1.01
Rat no. 3	1.17	147.00	0.80
Rat no. 4	1.37	136.00	1.01
Rat no. 5	1.18	138.00	0.85
Rat no. 6	1.40	157.00	0.89
Mean ±SD	**1.35 ± 0.17[a, c]**	**148.00 ± 14.64[a, d, e]**	**0.91 ± 0.08[b]**
Preventive			
Rat no. 1	1.43	191.00	0.75
Rat no. 2	1.51	147.00	1.03
Rat no. 3	1.73	197.00	0.88
Rat no. 4	1.63	190.00	0.86
Rat no. 5	1.70	187.00	0.91
Rat no. 6	1.57	163.00	0.96
Mean ±SD	**1.59 ± 0.11[c]**	**179.17 ± 19.66[b, d, f]**	**0.90 ± 0.09[c]**
Treatment			
Rat no. 1	1.47	101.00	1.45
Rat no. 2	1.44	165.00	0.87
Rat no. 3	1.57	105.00	1.49
Rat no. 4	1.28	103.00	1.24
Rat no. 5	1.53	121.00	1.26
Rat no. 6	1.52	119.00	1.28
Mean ±SD	**1.46 ± 0.11[b]**	**119.00 ± 26.91[c, e, f]**	**1.27 ± 0.25[a, b, c]**

*The difference is significant at p < 0.05 level (a, b, c, d, e, f)

no significatly different ratio compared to the control and diabetic rats (p > 0.05). In contrast, the rats treated by nettle extract after diabetes induction showed the highest ratio among the experimental groups (p < 0.05).

The area densities of granule cells in the four groups of the experiment are shown in Table 2. The ANOVA test showed that there were no significant differences in the granule cell densities of the four groups (p > > 0.05). Considering the homogeneity of variances, the LSD test was chosen for multiple comparisons between pairs of the groups. The granule cell densities of the diabetic and preventive groups were lower than that of the control group, but no statistical significance was found (p > 0.05). The treatment group showed a higher density than the diabetic group (p > 0.05). In addition, the cell density of the treatment group was significantly higher than that of the preventive group (p < 0.05). Interestingly, no significant difference was

Table 2. The area density of granule cells in the four experimental groups

	Area density (count of granule cells/μm^2)	Standard deviation	Standard error of mean
Control	7356×10^{-6}	2118×10^{-6}	299×10^{-6}
Diabetic	6896×10^{-6}	1564×10^{-6}	233×10^{-6}
Preventive	6804×10^{-6a}	1412×10^{-6}	187×10^{-6}
Treatment	7582×10^{-6a}	2323×10^{-6}	350×10^{-6}

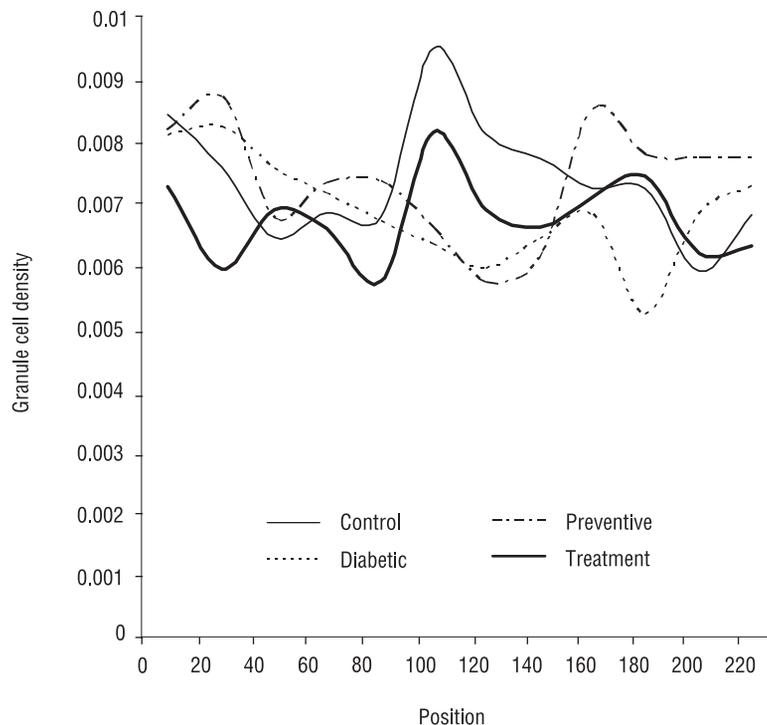a — the difference is significant at $p < 0.05$

Figure 3. The anterior-posterior transitions of granule cell density in the four experimental groups; position is the distance [μm] of each section from the reference point. Granule cell density is the cell number in 1 μm^2. See the text.

found between the densities of the control and treatment groups ($p > 0.05$).

The individual correlations between granule cell density and the anterior-posterior position of the section, mean glucose level at 5 weeks and rat brain weight were examined by the Pearson correlation test.

When the data from all 24 rats were considered, it was apparent that the granule cell density was not correlated with the anterior-posterior position in which the density were measured (R = –0.095; $p > 0.05$). In contrast to this finding, the diabetic group showed a significant correlation between these variables (R = –0.464; $p < 0.05$).

The Pearson correlation test showed controversial correlations between granule cell density and brain weight. Taking the 24 as a whole, there was

a negative correlation with no statistical significance between these two variables (R = –0.014; $p > 0.05$), whereas the treatment group showed a positive and significant correlation between them (R = 0.394; $p < 0.05$). The diabetic and control groups showed a significant negative correlation between granule cell density and brain weight (R = –0.322, –0.395 respectively; $p < 0.05$).

The granule cell densities of the rats in total and their mean glucose levels during 5 weeks of study were not correlated (R = –0.024; $p > 0.05$). In addition, only the protective group showed a significant correlation between them (R = –0.299; $p > 0.05$).

The anterior-posterior transitions of granule cell density in the four groups of the experiment are shown in Figure 3. The horizontal axis shows the

Table 3. Multi-variable regression models showing the net effects of different factors on the granule cell density in different experimental groups

Total	$D = 0.007 - 2.41 \times 10^{-6} P$	$P > 0.05; R^2 = 0.009$
Control	$D = 0.030 - 0.013 B$	$P < 0.05; R^2 = 0.156$
Diabetic	$D = 0.008 - 9.07 \times 10^{-6} P$	$P < 0.05; R^2 = 0.215$
Preventive	$D = 0.008 - 3.74 \times 10^{-6} G$	$P < 0.05; R^2 = 0.089$
Treatment	$D = -0.005 + 0.009 B$	$P < 0.05; R^2 = 0.155$

The significance was considered at $p < 0.05$; D — granule cell density; P — anterior-posterior position; G — mean glucose level of the 5 weeks; B — rat brain weight; R — coefficient of correlation.

relative anterior-posterior position of the sections from the reference position (i.e. Bregma –3.30 mm). The vertical axis represents the area densities of granule cells corresponding to the positions.

The transitions showed that the anterior-posterior distributions of granule cell density were not the same in the four groups, but some similarities between control and treatment groups were seen which could support the density results mentioned earlier. In addition, no significant regression model, including linear, quadratic and logarithmic, was compatible with the transitions drawn up ($p > 0.05$). It is difficult to imagine an anterior-posterior trend of granule-cell susceptibility to diabetes according to positional variability in the cell density.

The net effect of the variables, including the anterior-posterior position of the section (P), mean glucose level at 5 weeks (G) and rat brain weight (B) on the density (D), were shown in each of four groups using multi-variable regression models by the "backward" method (Table 3).

By the regression model, multi-variable equations such as $D = a_0 + a_1P + a_2B + a_3G$, were provided for the total sample as well as for each group. The backward method tested the statistical significance of each component in all the suggested equations and finally proposed the most significant equations. As shown in Table 3, no uniform models were found with all three variables within one equation. This demonstrates that a significant multi-factorial model showing the net effect of the different variables on the density was not achieved either for the total sample or for each group. In addition, the effects of the variables on the density were not the same in the four groups.

DISCUSSION

The results of the present study showed a decrease in granule cell density following STZ-induced diabetes which was not statistically significant. In addition, the results suggest that the preventive administration of *U. dioica* extract used in this study showed no neuroprotective effect on granule cell loss in diabetic rats. In contrast, treatment with nettle extract in STZ-induced diabetic rats can help to restore the neuronal loss which is observed in diabetes.

The decrease in cell density seen in the diabetic group was compatible with the results of previous studies. For example, Beauquis et al. [2] showed that neurons counted in the hilus of the dentate gyrus decreased by 30% in diabetic mice compared with controls. An explanation for this decrease in cell density can be the diminished proliferation of precursor neurons and new neuron survival, which have been demonstrated by various studies [2, 13, 24, 29]. In addition, involvement of cholinergic dysfunction, oxidative stress and inflammation in the development of cognitive impairment in diabetic animals are factors that have been proposed by some studies [1, 7, 18].

The rats of the treatment groups showed no evidence of granule cell loss, indicating that therapeutic use of nettle extract may have significant regenerative effects on the granule cells and their progenitors. It is now well documented that new neurons produced in the subgranular zone arrive into granule cell layer of the dentate gyrus [10]. It has been suggested that granule cell death stimulates the proliferation of precursor cells, many of which survive and differentiate into mature granule neurons and restore the granule cell loss in the dentate gyrus [11]. Given the results of the treatment group, it seems that nettle extract may enhance the production of the new neurons and compensate for the diabetic-induced granule cell loss.

The proposed neuroprotective property of nettle extract can be attributed to the antioxidant

activity of *U. dioica*. Increasing evidence suggests that oxidative stress plays an important role in diabetic neuropathology [1, 7, 18]. On the other hand, the antioxidant activity of *U. dioica* has also been shown by some studies [12, 22, 26]. These properties include inhibition of fatty acid peroxidation, effective reducing power, free radical scavenging, superoxide anion radical scavenging, hydrogen peroxide scavenging, and metal chelating activities, making the nettle extract a powerful natural antioxidant material [12]. In addition, the level of free electron accumulation in several rat brain areas, such as the right frontal lobe, has fallen following nettle leaf supplementation [22, 26]. In conjunction with its antioxidant activity the nettle extract can also up-regulate AP-1 and may have an anti-apoptotic and cell-survival supporting effect [26]. Given these properties, nettle extract may prevent oxidative injury and apoptotic cell death within the granule cell layer and subgranular zone of the dentate gyrus. Consequently, a lower number of mature and new granule cells would degenerate. In addition, the possible granule cell loss may be compensated for by production of new granule neurons within the subgranular zone.

It was apparent that the preventive and therapeutic administration of nettle extract can have different outcomes. The preventive group of rats had the lowest granule cell density, and granule cell loss similar to that of the diabetic group was found in these rats. In contrast, the rats of the treatment group had the highest granule cell density and there was no diabetic-induced granule loss. To explain this finding, it should be borne in mind that the prevention was limited to a short period before the diabetes; but the treatment was continued for 28 days after STZ-induced diabetes. The prevention period may therefore be too short for the neuroprotective effects to appear. In addition, the preventive nettle extract was administered before the diabetes-induced loss had been established. As mentioned earlier, it has been shown that new neuron production to reverse the granule cell loss is a lesion-induced phenomenon [11]. As a result, it seems that prevention may not enhance the compensatory neurogenesis, as discussed earlier with regard to therapeutic intervention.

In conjunction with its main results, this study provided some supplementary data including GTT, brain weight, correlations and transitions. The GTT results, reflecting the insulin secretion states, support the preventive approach, as mild impairment was seen in the preventive group. This finding was not compatible with results regarding density which confirm a neuroprotective role for the treatment. On the other hand, a significant negative correlation between mean glucose level and density was found in the preventive groups but not in the treatment group. These findings together may suggest that the therapeutic effects of the nettle extract observed are not necessarily mediated by an improvement in insulin secretion and hyperglycaemic state.

The results show that brain weight was not a specific indicator for the neuroprotective outcomes of nettle administration; because it can be affected by the body weight of the animal. To make a more precise comparison, an attempt was made to omit the effects of the body weight as a confounding variable and the brain/body weight ratio were proposed. The results showed that the treatment groups had the highest ratio, which may support the treatment rather than the prevention. However, it may not be specific either, because the changes in body weight can affect the results.

An attempt was made to find the anterior-posterior transitions of the density and provide the mathematical equations for computational analysis. Transition of the cell density can show the distribution of the density in anterior-posterior transition. Moreover, the anterior-posterior trend of susceptibility can be discovered. In a similar manner, the multi-variable equation can show the net effect of all the variables on the density.

The underlying trends of the transitions drawn up were not significantly describable by regression models, which show that the behaviour of the trait may be compatible with different models provided by neural network methods. Similarly, the results observed showed a considerable heterogeneity between the different groups and this was reflected by the lack of uniform equations. It was difficult therefore to propose a comprehensive multi-variable equation to represent the net effect of the all the variables on the density.

CONCLUSION

The study showed that *Urtica dioica* extract can help to restore diabetes-induced granule-cell loss in the rat dentate gyrus. However, preventive use of the extract showed no significant benefit. It seems that nettle extract administration can be a beneficial treatment for the diabetic neuropathology in the dentate gyrus and that this can ameliorate cognitive impairment in the diabetic patient.

ACKNOWLEDGEMENTS

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