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High fructose solution induces neuronal loss in the nucleus of the solitary tract of rats

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Abstract

Nucleus tractus solitarius (NTS) is the main region for the cardiovascular regulation including the baroreceptor reflex. Although the physiological and biochemical aspects of a high fructose diet on the brain have been studied, its effects on the quantitative aspects of the NTS have received less attention. The objective of the present study was to quantify the structural changes in the NTS after the consumption of high fructose solution, using stereological methods. Male rats were distributed randomly into two groups. One group received fructose (10%) in tap water for six weeks and the other one received tap water without fructose. The total volume of the NTS and number of the neurons of the nucleus were estimated using stereological methods including Cavalieri and disector methods. The coefficient of error (CE) of the estimators was calculated. Fasting plasma concentrations of glucose, triglyceride, cholesterol and insulin were obtained. Also a glucose tolerance test was done. The results showed no significant changes in glucose, triglyceride, cholesterol and insulin concentrations between the two groups. The glucose tolerance test did not show any differences, either. The mean (coefficient of variation) volume of the NTS and the neuronal number was 0.68 (0.2) mm^3 and 68000 (0.13) in the control rats, respectively. But the volume of the NTS and total number of the neurons decreased by 41% in the fructose-treated group ($p \leq 0.01$). Consumption of high fructose solution for six weeks led to a decrement in the volume and number of the neurons in the NTS in rats.

Key words: *nucleus tractus solitarius, fructose, stereology, rat.*

Introduction

Simple sugars such as fructose with saturated fats are believed to be the major components of diet in many societies [27]. Carbohydrated soft drinks sweetened with fructose comprise about 33% of the total daily sugar intake in some drinks and foods. Fructose is used in several forms including high fructose corn syrup, sucrose, carbonated beverages, fruit products, cereals and other dairy products [20]. Unhealthy dietary habits can result in a heavy toll on the brain health. Overconsumption of fructose, particularly in the form of soft drinks, is increasingly [2] recognized as a public health concern [8]. A high fructose diet causes numerous pathological changes including oxidative stress, obesity, metabolic syndrome, glucose intolerance, insulin resistance, type II diabetes, liver disease, hypertension and cardiovascular disease [25].

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It has been shown that consumption of fructose solution (15%) and a high fructose diet for six weeks can lead to an impaired cognitive function [2,22]. An intact plasma membrane is required for growth, repair and synaptic plasticity [2]. Researchers have claimed that fructose intake disrupts the plasma membrane of the neurons and the neuronal function might be affected [2]. It has been shown that consumption of a high caloric diet has harmful consequences for synaptic plasticity [31]. Also, a high caloric diet impairs the cognitive function, memory, dendritic spine density, synaptic plasticity [26,27] and neurogenesis in the hippocampus [30].

Although human and animal studies have proposed that excessive energy intake affects some regions in the brain adversely [27], the nucleus of tractus solitarius (NTS) has received less attention.

The NTS is a region in the brain that regulates and is involved in baroreflex sensitivity. Previous studies showed that the diet containing fructose could affect the morphology of the NTS. It was shown that a high fat and high carbohydrate diet taken for 30 days induced structural changes in the NTS and decreased the sensitivity of the baroreceptor reflex [3]. The structural changes were observed using transmission electron microscopy and including medullary sheath thickening, myelinated nerve atrophy and hyaloplasm dissolving. It has been shown that consumption of large quantities of fructose results in the impairment of baroreflex sensitivity and neurogenesis. Although the physiological and biochemical aspects of a high fructose diet on the brain have been previously studied, its effects on the quantitative aspects of the NTS have received less attention. The aim of the present study was to quantify the structural changes in the NTS following the consumption of high fructose solution in male rats using stereological methods.

Material and methods

Animals

Male Sprague-Dawley (150-180 g) 2.5-month-old rats were selected from the animal lab of the Shiraz University of Medical Sciences. All animal experiments were approved by the Animal Ethics Committee (approval license 90-5702) and kept in accordance with the university guidelines. Animals were weighed and distributed randomly into two groups (n = 6). They were kept at standard temperature (22-25°C) and in 12 h light-dark cycles. The rats had free access to tap water and fructose solution (10%) in the control and fructose-treated groups, respectively, for 6 weeks.

Intraperitoneal glucose tolerance test

On the last day of the experiment, the animals were kept fasting for 12, 0, 0 h and then anesthetized with ketamine and xylazine (70 and 5 mg/kg i.p., respectively). An unchallenged blood sample was checked at time of zero from the tail. Thereafter, 2 g/kg glucose was injected intraperitoneally and after 15, 30, 60, 90 and 120 minutes, blood samples were checked by the glucose meter (ACCU-CHEK Active, Germany) to determine the glucose concentration.

Biochemical analysis

After performing the glucose tolerance test, a sample of 2 ml of blood was obtained. Blood samples were centrifuged at 3000 g for 10 min to obtain the plasma samples; then, glucose, triglyceride and cholesterol were quantified by the enzymatic method (Pars Azemoon Kit, IRAN) and plasma insulin level was assayed by rat insulin ELISA kit (Mercodia, Sweden).

Estimation of volume of the NTS

The brain was removed after transcardial perfusion-fixation with 2% paraformaldehyde in 0.1 M sodium phosphate buffer (pH: 7.2) for 20 min [23]. After histological processing, the brain stem was embedded in paraffin block.

The volume of the left NTS was estimated using Cavalieri method. Briefly, the rat medulla was sectioned exhaustively into 25 µm thick sections (t) using a microtome, from –11.04 to –15.96 mm from bregma [14,24]. About 8-12 sections in each animal were selected in a systematic random manner with a known fraction (k) (Fig. 1). The sections were stained with Cresyl violet. Using a microscope connected to a camera, the live image of each section was evaluated according to the rat brain atlas [24] at final magnification of 23× using stereological software designed at Histomorphometry & Stereology Research Centre (Shiraz University of Medical Sciences). The sum of the area of the sections ($\sum A$) of the NTS was estimated using the software designed at the centre (Histomorphometry & Stereo-

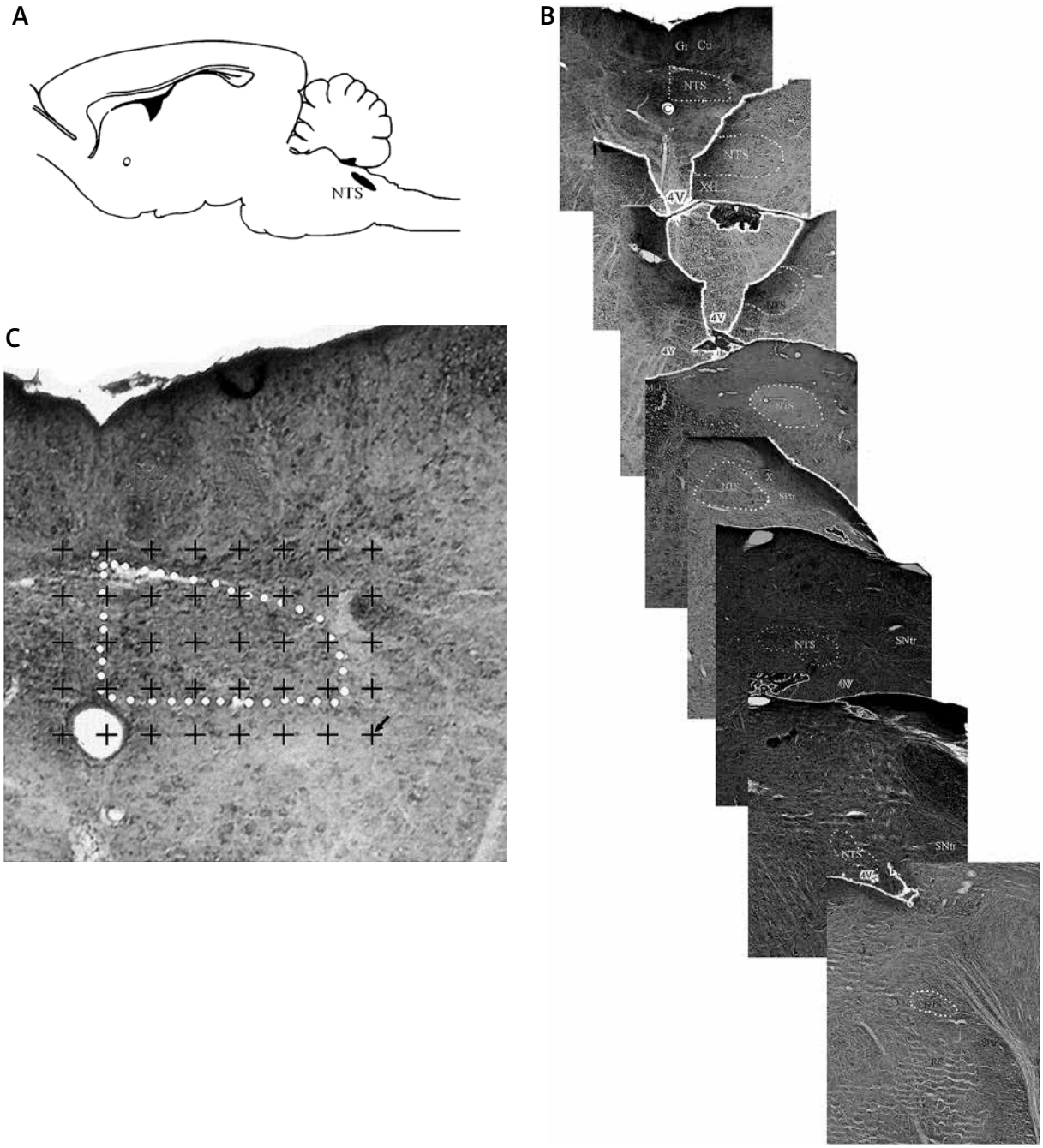

Fig. 1. A) A schematic drawing of the rat brain which shows the location of NTS. **B)** Sampling of the sections and determination of the NTS boundary. An exhaustive collection of the transverse sections of the brain stem were obtained and eight-twelve systematic random sections were sampled (here 8) for estimating of the NTS volume using Cavalieri method. The boundaries of the nucleus tractus solitarius, fourth ventricle (4V), cuneatus (Cu), gracilis (Gr), medial longitudinal fasciculus (mLF), twelve nerve (XII), rhinal fissure (RF) and spinal trigeminal nucleus (SPn) are marked. **C)** The area of each section of the NTS was estimated using the point counting method.

logy Research Centre, Shiraz University of Medical Sciences). In addition, V(NTS) was estimated by the sum of points multiplied by the area per point using a point-counting method (Fig. 1). The area per point (*a/p*) was 0.02 mm^2 and 180 and 185 total points were counted on average per animal in the control

and fructose-treated groups, respectively. Finally, the volume was estimated by:

$$V(NTS) = k \times t \times \sum A_{(NTS)}$$

In the case of the second method, the formula was:

$$V(NTS) = k \times t \times \sum P_{(NTS)} \times (a/p)$$

Estimation of the total number of neurons

The numerical density N_V (cell/NTS) or number of neurons in each unit volume of the NTS was estimated using the optical disector method, the position of the microscopic fields was selected by systematic uniform random sampling. An oil immersion on objective lens (100×) was used. An unbiased counting frame with inclusion and exclusion lines was superimposed on the images at final magnification of 3400× (Fig. 2). This frame avoids the "edge effect" and biased counting of the particles. The focal plane was moved downwards in z direction. A microcator was attached to the microscope to measure the z-axis travelling (depth). The guard zones were used to avoid cutting artifacts that occur on the upper and lower surfaces of the sections. Any counting event in focus within the upper (the first 5 µm) or lower guard zones was ignored. The distance between the guard zones was the "height of disector" which was 15 µm here. Any nucleolus coming into the maximal focus within the next focal sampling plane was selected if it was located completely or partly inside the counting frame and did not touch the exclusion line (Fig. 2). The numerical density (N_V) was estimated using the following formula:

$$N_V(cell/NTS) = \Sigma Q^{-}/(\Sigma p \times a/f \times h) \times (t/BA)$$

here ΣQ^{-} was the number of the nucleoli coming into focus, Σp was the total counting of the unbiased counting frame in all fields, and *h* was the height of dissector. A point (P) lying at the centre of the counting frame was used to facilitate the counting of the frame area hitting the reference space. On the average, 160-400 cells were counted per NTS. Where *a/f* was the frame area, *t* was the real section thickness measured using the microcator, and *BA* was the block advance of the microtome which was set at 25 µm. Although in the present work we did not calibrate the microtome, it has been advised to calibrate the microtome to do a better evaluation [11]. The section thickness was measured at 6-10 fields of vision by sampling uniformly at random from each section and 72-120 location per animal. The mean section thickness was 20.3 µm in both groups. The total number of the neurons was estimated by multiplying the numerical density (Nv) by the V(NTS) [15,16,19].

Estimation of the coefficient of error (CE)

The CE for the estimate of the volume, that is CE(V), is the function of the noise effect and systematic random sampling variance for the sums of areas.

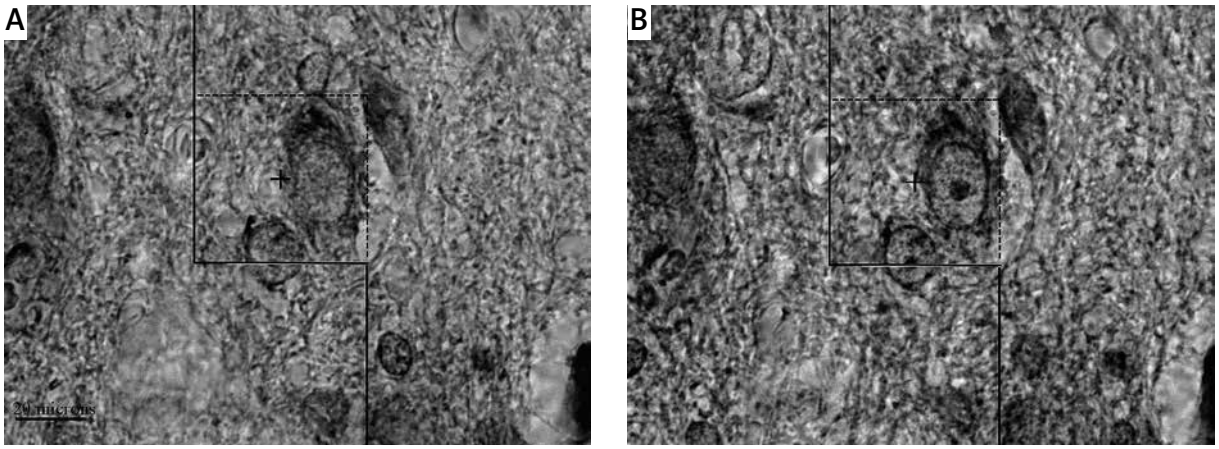

Fig. 2. Dissector method. The nucleolus of the neuron is considered as a criterion for counting. Any nucleolus that is not in focus at the beginning of the dissector height **(A)** and is coming into maximal focus within the focal sampling plane **(B)** is selected. It must be located completely or partly inside the counting frame and not touch the exclusion line.

When the cross-sectional areas "∑A" were estimated by the software, CE(V)was calculated using the following formula [17,18]:

$$CE(V) = (\sum A)^{-1} \times [1/12 \times (3\sum A_i A_i + \sum A_i A_{i+2} - 4\sum A_i A_{i+1})]^{1/2}$$

When the cross-sectional areas were estimated by point counting, CE(V) was calculated using the following formula [17,18]:

$$CE(V) = (\sum P)^{-1} \times [1/240\ (3 \times \sum P_i P_i + \sum P_i P_{i+2} - - 4\sum P_i P_{i+1}) + 0.0724 \times b/a^{1/2} \times (n\sum Pi)^{1/2}]^{1/2}$$

Where *b* and *a* represent the mean section boundary length and mean sectional area, respectively. The CE for the estimate of the total neuron number, that is CE(N), was derived from CE(V) and CE(Nv) as follows [6]:

$$CE(N) = [(CE^2(Nv) + CE^2(V)]^{1/2}$$

$$CE(Nv) = [(n/n - 1) \times [(\sum(Q^-)^2/\sum Q^- \sum Q^-) + + (\sum(P)^2/\sum P \sum P) - (2\sum (Q^- P)/\sum Q^- \sum P)]]^{1/2}$$

Statistical analysis

Data were collected, analysed, and reported as mean, standard deviation (mean ±SD) and coefficient of variation (CV). Kruskall-Wallis and Mann-Whitney *U*-test and independent *t*-test were used to compare the differences between the groups. A $p \leq 0.05$ was considered as statistically significant.

Results

Body and brain weights

The data showed no significant changes of these parameters in the fructose-treated rats in comparison to the controls (Table II).

Fluid intake

Analysis of data during 6 weeks showed that there was no difference between the two groups.

Biochemical analysis

The glucose, insulin, triglyceride and cholesterol did not show any significant differences between the two groups (Table I).

Glucose tolerance test

The data showed no significant difference between the two groups.

Volume of the NTS

The volume of the nucleus tractus solitarius was decreased by 41% on the average in fructose-treated rats when it was compared to the control groups ($p \leq 0.01$) (Table II, Fig. 3).

Total number of the neurons

The results showed that the total number of the NTS neurons was significantly decreased 41%

Table I. Mean ±standard deviation and on the plasma levels of glucose (mg/dl), triglyceride (mg/dl), cholesterol (mg/dl) and insulin (µg/l) in control and fructose-treated rats

Groups	Glucose	Triglyceride	Cholesterol	Insulin
Control	132.2 ± 5.1	97.6 ± 11.8	68.8 ± 2.3	0.33 ± 0.10
Fructose	135.6 ± 4.7	86.0 ± 6	72.6 ± 3.7	0.46 ± 0.17

Table II. Mean ±standard deviation of the body and brain weights (g) (at the end of the experiment), total volume (mm^3) of the NTS and number of the neuron of the NTS in control and fructose-treated groups. Coefficient of variation (CV) and coefficient of error (CE) are presented for stereological estimations

	Weight		NTS volume	Neurons number
Groups	Body	Brain		
Control (CV)(CE)	273 ± 17	2.5 ± 0.2	0.68 ± 0.17 (0.20)(0.06)	68000 ± 9000 (0.13)(0.12)
Fructose (CV)(CE)	287 ± 22	2.7 ± 0.2	0.40 ± 0.05* (0.12)(0.05)	39000 ± 6000* (0.15)(0.14)

**p < 0.01, Control vs. fructose-treated rats*

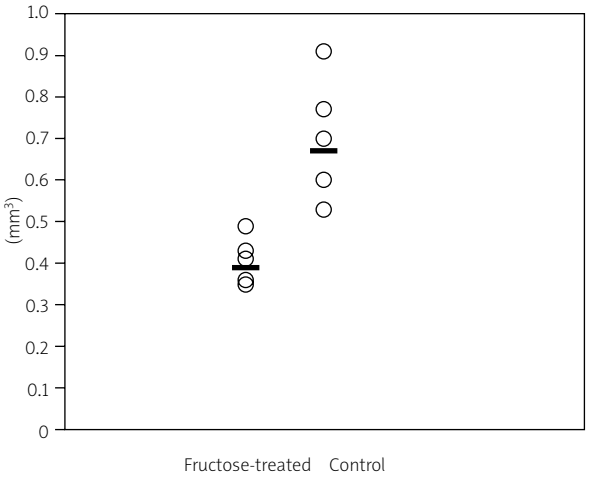

Fig. 3. Total volume of the NTS in the control and fructose-treated rats.

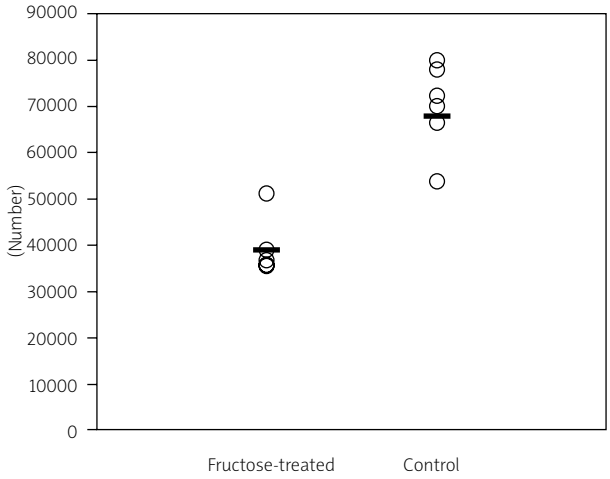

Fig. 4. Total number of the neurons in the NTS of the control and fructose-treated rats.

on the average in the fructose-treated rats in comparison with the control groups ($p \leq 0.01$) (Table II, Fig. 4).

Coefficient of error

There is no difference between the prediction of coefficient of error (CE) for volume estimation of the NTS using the two described methods (areas estimation and point-counting methods).

Discussion

The present study evaluated the effects of consumption of high fructose solution for 6 weeks on the volume and number of neurons in the NTS of rats. The main goal of the present study was to assess the structural change of the NTS. The advantage of using stereological studies is obtaining unbiased and accurate estimations.

The weight of the animal and liquid intake were the first evaluated parameters. Some previous studies by Stranahan *et al*. and Brito *et al*. reported that high fructose diets induced weight gain and more drinking liquid in rodents [7,21,27]. Contrary to their claim, in this study, no weight gain and change in liquid intake was observed. The finding of the present research is in the same line with those of Takatori *et al*., Van der Borght *et al*. and Zamami *et al*. [28,30,32]. A probable description might be a different route of administration. Administration through drinking water may cause fewer amounts of fructose intake than food. Another reason might be a different species of experimental animals. Different species might show different levels of susceptibility after fructose treatment as a weight-gainer sugar. No difference was observed in the body weight, suggesting that obesity is not a major contributor to altered structural changes in the NTS.

Contrary to the findings of Takatori *et al*., Brito *et al*., Abdulla *et al*. and Catena, when consumed in drinking water in rats and hamster, fructose did not cause a significant impairment in glucose tolerance; this is in the same line with the results of the present study [1,7,9,28]. As listed earlier, different administration routes and different species of animals may explain the controversy observed [6,21,29].

In accordance with the present results, Van der Borght *et al*., Ueno *et al*. and Axelsen *et al*. [5,29,30] showed that consumption of fructose could not affect the insulin, free fatty acid and glucose levels in fasting state. The controversial findings are reported as well [4,7,27-29,32]. Alterations in circulatory parameters are accompanied by an increase in body weight since no difference was observed in the body weight; thus, no alteration in free fatty acid, triglyceride, fasting glucose and insulin is predictable.

The present study showed that the consumption of high fructose solution for 6 weeks reduced the volume of the NTS and number of the neurons without insulin resistance. The damaging effects of a high fructose diet extend directly to the brain [22], impairing the spatial memory in rats [25]. It was shown that

male rats consuming fructose, in particular, have an increased amount of apoptotic cells in the dentate gyrus of the hippocampus and the impairment in neurogenesis [30]. However, by induction of insulin resistance, the cognitive function, structural plasticity and hippocampal function are impaired and dendritic spine density is reduced [27].

However, in the present study no change was seen in the insulin level. Therefore, other mechanisms can be responsible for the neuronal loss. Evidence is accumulating that neuronal cells can metabolize fructose. Thus, it is possible that fructose directly affects the brain as reported by Funari *et al*. [13]. The high fructose diets induce the toxic effects (e.g. hypophosphatemia, hyperuricemia) due to high fructose concentrations. Nocturnal hypertension and sympathetic nervous system changes due to a high fructose diet have been reported [12]. Impaired neurogenesis in the hippocampus by Van der Broght *et al*. is also reported [30]. Based on the results of these studies, fructose or one of its metabolites might induce loss of neurons in the NTS. Further research is suggested to assess the mechanism of fructose action on the neurons.

The modern stereological methods were applied to estimate the NTS volume and the numerical density of the neurons including Cavalieri and disector techniques. There are a limited number of research to show the volume of the NTS and their neuron number in rats. Dentremont *et al*. reported that the volume of the NTS was ~0.16 mm^3 in the mouse [10]. They also reported that the total number of the neurons was ~87 000. They have used a modification of the Abercrombie method in contrast to our research which has relied on modern stereological methods.

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