CrossMark

ORIGINAL PAPER

Neuroprotective effects of *Nigella sativa* extract upon the hippocampus in PTU-induced hypothyroidism juvenile rats: A stereological study

Farimah Asiaei[1] · Alireza Fazel[1,2] · Ali Akbar Rajabzadeh[1,2] · Mahmoud Hosseini[3] ·
Farimah Beheshti[4] · Masoumeh Seghatoleslam[1,2]

Abstract This study aimed to examine the neuroprotective effects of *Nigella sativa* (*N. sativa*) in the hippocampus of propylthiouracil (PTU)-induced hypothyroid rats during neonatal and juvenile growth. Twenty- five pregnant rats from early gestation (GD 0) were divided into five groups: (1) control (received drinking water), (2) PTU (received 0.005% PTU in drinking water), (3–5) PTU + NS 0.05%, PTU + NS 0.1%, PTU + NS 0.2% (along with PTU, received 0.05%, 0.1% and 0.2% *W/V* of *N. sativa* respectively) and treatment continued until postnatal day 60 (PN 60). The brains of male pups were removed for histological and stereological assessments. *N. sativa* extract significantly reduced the production of dark neurons and apoptotic cells in different areas of the hippocampus compared to the PTU group. Moreover, it significantly attenuated the effect of hypothyroidism on the volume reduction of the hippocampus. The results of the present study suggested that *N. sativa* extract has a potential ability to prevent the hippocampal neural damage after inducing hypothyroidism during neonatal and juvenile growth in rats.

✉ Masoumeh Seghatoleslam
    seghatoleslamm@mums.ac.ir; anita49b@yahoo.com

1   Department of Anatomy and Cell Biology, School of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran

2   Microanatomy Research Center, Mashhad University of Medical Sciences, Mashhad, Iran

3   Division of Neurocognitive Sciences, Psychiatry and Behavioral Sciences Research Center, Mashhad University of Medical Sciences, Mashhad, Iran

4   Neurogenic Inflammation Research Center, Mashhad University of Medical Sciences, Mashhad, Iran

Introduction

The thyroid gland is one of the most important endocrine glands that it's hormones; thyroxine (T4) and Triiodothyronine (T3) have an important role in the regulation of body metabolism, physical and mental development, maturation and reproduction (Wagner et al. 2008). Moreover, they are crucial in neural generation and development of nervous system during fetal and neonatal life (Legrand 1984; Shin et al. 2013). Hypothyroidism, which is most common chronic diseases in the world are related to insufficient production and secretion of two thyroid hormones (TH) (Jannini et al. 1995). Late diagnosis and untreated congenital hypothyroidism can lead to a number of severe health problems in neonates such as cognitive and neurological impairments as well as physical abnormalities known as Cretinism (Ford and Cramer 1977). Former studies have shown that maternal hypothyroidism during both neonatal and lactation periods influences the development of the brain and can cause learning and memory impairments and structural abnormalities in rodents (Cragg 1970; Morreale de Escobar et al. 2004). It is well documented that this memory and learning impairment due to thyroid hormone deficiency leads to cell reduction in the granular layer of dentate gyrus as well as the pyramidal layer of CA1 region in the hippocampus (Madeira et al. 1992; 1991). Furthermore, prenatal hypothyroidism can reduce dendritic spines in the hippocampal pyramidal cells and impairs synaptic function in CA1 and dentate gyrus (Gong et al. 2010; Shibutani et al. 2009). It seems that the hippocampal formation is particularly sensitive to thyroid hormones and developmental hypothyroidism can cause anatomical changes in the hippocampus (Madeira et al. 1992). Former studies have revealed that hypothyroidism can lead to a reduction in the size of the hippocampus in the human brain and it has been shown that hippocampal volume reduction provides learning and memory disorders (Cooke et al. 2014).

According to the previous studies, the cell loss and neural damages following the hypothyroidism can be related to the production of dark neurons (Madeira et al. 1991) and apoptotic cells (Sinha et al. 2009) in the hippocampus. Dark neurons which appear such as the shrunken and hyperchromatic cells with small dense nuclei and hyper electron density properties occur under different conditions such as seizure, ischemia, head injury or an electric shock as well as metabolic disorders like hypoglycemia (Zsombok et al. 2005). On the other hand, apoptosis is a process of programmed cell death occurs in physiological conditions which characterized by specific morphological features including cytoplasmic shrinkage and chromatin condensation and segregation (Ataei and Ebrahimzadeh-Bideskan 2014). A variety of internal or external stimuli such as free radicals, radiation, chemotherapy, growth factors deprivation, calcium influx, and death ligands promote the apoptosis (Bratton et al. 2000). The cause of induced memory impairment in hypothyroidism has not been known exactly. However, it is well known that among the various hormonal influences that work on the antioxidant regulation, thyroid hormones play particularly important roles and they can modulate antioxidants in humans and animals (Mancini et al. 2016; Yilmaz et al. 2003). Besides, some studies have demonstrated that hypothyroidism-induced oxidative stress in the nervous system results in memory impairment (Cano-Europa et al. 2008).

Many plants- derived medications employed by the ancients are still in use today and many medicinal plants containing antioxidants were appointed to treat a variety of ailments (Gupta and Sharma 2006). *Nigella sativa,* commonly known as the black seed, is an annual plant (Family Ranunculaceae) that widely used in folk medicine throughout the world to treat a number of diseases and conditions such as asthma, diarrhea, hypertension, diabetes, inflammation, headache and fever. The most important bioactive compounds of *N. sativa* are thymoquinone, alkaloids, riboflavin, piridoksin, niacin, folic acid, minerals and proteins (Jalali and Tehranipour 2013; Salem 2005). It has been shown that thymoquinone, the major component of this plant inhibits non-enzymatic lipid peroxidation in liposomes and has cytoprotective and antioxidant effects (Ali and Blunden 2003). In addition, *N. sativa* oil has preventive effects on lipid peroxidation during cerebral ischemia injury in rat hippocampus (Hosseinzadeh et al. 2007). Regarding this antioxidant effect of *N. sativa*, the numerous in-vivo and in-vitro studies suggest that *N. sativa* has neuroprotective effects in neurodegenerative disorders (Islam et al. 2015; Sedaghat et al. 2014). Moreover, the findings of previous studies have shown that this plant can prevent hippocampal neuronal damage and improve the memory in pentylenetetrazole (PTZ) seizure models (Seghatoleslam et al. 2016). It has been revealed that administration of *N. sativa* increases thyroid hormone

concentrations in rats (Sharif et al. 2012). In addition, former studies showed that it could improve learning and memory during neonatal and juvenile growth in rats with hypothyroidism (Beheshti et al. 2017). Based on the antioxidant and neuroprotective properties of *N. sativa*, the aim of this study was to investigate the effect of the plant hydro-alcoholic extract on neuronal damage and the hippocampal volume in the hypothyroid rats during neonatal and juvenile growth.

Materials and methods

Animals

Twenty-five pregnant female Wistar rats (12 weeks old and weighing 230 ± 20 g) were maintained in separate cages at 22 ± 2 °C, in a room with a 12:12 h light: dark cycle, and food and water available ad libitum. Animal handling and all related procedures were conducted in accordance with Mashhad University of Medical Sciences, Ethical Committee Acts.

Plant extraction

Seeds of *Nigella sativa*, which were purchased from a local market, were identified at the herbarium of Pharmacy School, Mashhad University of Medical Sciences, Iran. The hydro-alcoholic extract was prepared as has been described previously (Vafaee et al. 2015). Powdered seeds (100 g) of *N. sativa* was milled and sieved then incubated in 300 ml of ethyl alcohol 70%, in a Soxhlet extractor. The extraction with ethanol was continued on the residue and then concentrated in a rotary evaporator and kept at −20 °C until being used. In order to obtain the desired concentrations, 0.5, 1, and 2 g of the extract respectively were dissolved in 1000 ml of drinking water (Beheshti et al. 2017).

Experimental procedure

Animal were randomly divided into five groups ($n = 5$ in each group) including: (1) control, (2) propylthiouracil (PTU), (3) PTU + NS 0.05%, (4) PTU + NS 0.1%, and (5) PTU + NS 0.2%. Control groups received drinking water, whereas animals in the PTU group received drinking water supplemented with 0.005% PTU (Sigma Co., St. Louis, USA) to induce hypothyroidism. The animals of experimental groups including PTU + NS 0.05%, PTU + NS 0.1%, PTU + NS 0.2% received drinking water supplemented with 0.005% PTU and 0.05%, 0.1%, and 0.2% *W/V N. sativa* extract, respectively. These experimental treatments started from early gestation (GD 0) for pregnant rats and continued for their pups during the lactation and infancy period. At day 60 post-delivery, eight male offspring of each group were randomly weighed and

considered for histological assessments. To assert of hypothyroidism, the serum T4 levels measured by radioimmunoassay.

Tissue processing

Animal pups were anesthetized with a high dose of ketamine and then transcardially perfused with 100 ml of cold saline, followed by 100 ml fixative solution [4% paraformaldehyde in 0.1 M phosphate buffered Solution (PBS), pH 7.4]. Brains were slowly taken out and kept in the same fixative solution for 48 h. After performing a routine histological tissue processing, brains embedded in paraffin blokes. Then, using a rotary microtome, 5 μ thick serial sections were cut in a coronal plane and mounted on slides (Seghatoleslam et al. 2016). Furthermore, in accordance with the atlas of Paxinos and Watson the boundary of the hippocampus was defined and then 10 equidistant slices of each block from brain per animals was selected and mounted on poly-L-lysine coated slides for TUNEL assay (Dityatev et al. 2004). The rest of slides were stained with toluidine blue to determine dark neurons and the volume of the hippocampus. The areas of the hippocampus were studied and photographed using a light microscope equipped with a high-resolution camera (BX51, Olympus, Japan).

Evaluation of cell apoptosis

TUNEL assay (Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labeling) by means of TUNEL Kit (Roche, Germany) was applied to detect apoptotic cells (Ataei and Ebrahimzadeh-Bideskan 2014). Primarily, poly-L-lysine sections were deparaffinized with the xylene, rehydrated in descending ethanol series and rinsed in 0.1 M PBS for 10 min. The endogenous peroxidase of specimens was inactivated by 3% H_2O_2 in methanol and subsequently, the tissue sections were washed with PBS 3 times for 5 min. Then the specimens were treated with proteinase K (Roche, 20 μg / ml in Tris buffer) for 30 min at room temperature. After washing with PBS, the slides were incubated in the labeling reaction mixture containing terminal deoxynucleotidyl transferase and the deoxynucleotide at 4 °C overnight. In the following day, all sections were rinsed in PBS and incubated in POD solution for 30 min at ambient temperature and then slides were washed with PBS for 3 min and eventually, immersed at 0.01% 3, 3'Diaminobenzidine (DAB) in 0.1 M PBS plus 0.01 H_2O_2 for 10 to 15 min at room temperature in dark. After extensive washing with tap water, sections were counterstained with Harris hematoxylin for 1 min and coverslipped. By this method, the apoptotic cell nuclei are identified through the presence of dark brown (Bagheri-Abassi et al. 2015). For positive control, sections were incubated with DNase I (3000 U/ml in 50 mM TrisHCl, pH 7.5, 1 mg/ml BSA) for 10 min at 15–25 °C to induce DNA strand breaks,

then TUNEL reaction was applied. Moreover, negative control sections were incubated with label solution only (without terminal transferase) instead of TUNEL reaction mixture (Ataei and Ebrahimzadeh-Bideskan 2014).

Stereological analyses

Volume measurements as well as, TUNEL positive cell and dark neuron estimation were performed in the right hippocampus of rats using the unbiased stereological principles (West and Gundersen 1990; Pourzaki et al. 2017). In order to quantitative analysis of dark neurons and TUNEL positive cells per unit area of hippocampal subdivisions, 20× and 40× magnification images respectively of each section were transferred to a computer equipped with the freely available software Image J using a 10,000 μm^2 counting frame (Seghatoleslam et al. 2013). The mean number (N_A) of neurons per unit area in CA1, CA3 and DG (dentate gyrus) regions of the hippocampus was calculated using the following formula (Rajabzadeh et al. 2011):

$$N_A = \frac{\Sigma Q}{a/f . \Sigma P}$$

The numerical density (N_v) of dark neurons in the hippocampus is calculated using physical dissector method. This method involved examining two serial sections of known distance (h) apart. All regions of the hippocampus were examined using 20× objective magnification and a minimum number of 10 pairs sections, with 10 μm distance were collected from each brain. The first section of each pair was appointed as the reference and the second one as look-up section. On each pair of sections, at least 10 microscopic fields were selected randomly in the area of interest and according to the unbiased frame counting rule, the estimation of dark neurons in each field was done using the following formula (West 1993).

$$N_V = \frac{1}{a/f.h} . \frac{\Sigma Q}{\Sigma P}$$

In mentioned formulae, "ΣQ" is the summation of counted neurons appeared in reference sections, "ΣP" is the sum of the numbers of frames counted, "a/f" is the surface area of each square of the counting frame and "h" is dissector height.

The total number of dark neurons (N) in any given hippocampus was estimated by multiplying the volume of the hippocampus (as determined by the Cavalieri method) with the corresponding numerical density (Khan et al. 2015).

$$N = N_V . V_{ref}$$

The volume of the right hippocampus for each brain was estimated according to the Cavalieri method on toluidine blue 5 μm equal distance sections. In this study, an average of 10

sections of the hippocampus was chosen and viewed at 4× magnification under a light microscope which is connected to a computer with Pro Plus image analyzer. Each image was superimposed at random, with a standard point grid. Then count how many points hit the subdivisions of the hippocampus in the sampled sections. The volume of any given hippocampus (V) was estimated by summing the areas associated with each point (a/p) and multiplying by the slice thickness (T) according to following the formula (Namavar et al. 2012).

$$V = T.a/p.\sum P$$

In this formula, $"\sum P"$ is the total number of points of the grid landing within the hippocampal region on the sections.

In this study, due to indicate the precision of the employed sampling methods, the Coefficient of Error (CE) was calculated (Namavar et al. 2012). In accordance with the Gundersen method (Chareyron et al. 2011) and using the following formula:

$$CE : \frac{(3a + c - 4b)^{\frac{1}{2}}}{2\sum p}$$

"$\sum P$" is the sum of the number of points in the squares counted in the sections used in the analysis; $A = \sum Pi^2$, $B = \sum Pi\, P\,(i + 1)$ and $C = \sum Pi\, P\,(i + 2)$.

Statistical analysis

All data were analyzed using analysis of variance (ANOVA) followed by Tukey's post hoc multiple comparison tests. Values are expressed as means ± standard error of the mean (S.E.M.) and $P < 0.05$ was considered statistically significant.

Results

Biochemical assessment

The serum T4 concentration in PTU group was significantly lower compared to that of the control group ($P < 0.001$, Table 1). Treatment by all three doses of the N. sativa extract significantly attenuated the PTU-induced alleviation in serum T4 ($P < 0.01$, Table 1).

The effect of Nigella sativa extract on body weight of hypothyroidism rats

Hypothyroidism which induced by PTU in the neonate and juvenile rats caused a significant reduction in body weight at PTU group compared to the control group ($P = 0.002$). Administration of N. sativa extract at the concentration of 0.05% W/V did not change the body weight of the animals ($P = 0.2$). However, consumption of 0.1% and 0.2% W/V of N. sativa extract

Table 1 Serum T4 concentrations in offspring. The T4 level in the PTU group was significantly lower than control, while administration of Nigella sativa at three concentrations significantly increased the level of T4 in the serum of all PTU-NS groups compared to PTU group

Group	Treatment	Serum T4 levels (μg/dl)
1	Control	7.1 ± 0.07
2	0.005% PTU	1.3 ± 0.23***
3	PTU+ 0.05% W/V NS	4.9 ± 0.19++
4	PTU+ 0.1% W/V NS	5.18 ± 0.06++
5	PTU+ 0.2% W/V NS	5.4 ± 0.04++

*** $P < 0.001$ compared to control group

++ $P < 0.01$ compared to PTU group

significantly increased the body weight of the rats in treatment groups compared to PTU group ($P < 0.05$; Fig. 1).

The effect of Nigella sativa extract on the apoptotic cells production

The apoptotic cells, which are presented in the CA1, CA3, and DG, identified as cells with a dark brown nucleus and irregular cytoplasm (Fig. 2). Few apoptotic cells were observed in the hippocampus of the control group, but hypothyroidism induced by drinking of PTU significantly increased the number of apoptotic cells in CA1, CA3 and DG regions of hippocampus compared to the control group ($P < 0.001$; Fig. 3). Administration of 0.05% W/V N. sativa extract significantly decreased the mean number of apoptotic cells in DG ($P = 0.006$) compared to PTU group. However, it did not change the number of these cells in CA1 and CA3 regions (Fig. 3). Treatment of the animals with 0.1% W/V of N. sativa extract significantly decreased the apoptotic cells in CA1, CA3 and DG regions in comparison to PTU group ($P < 0.01$ and $P < 0.001$ respectively; Fig. 3).
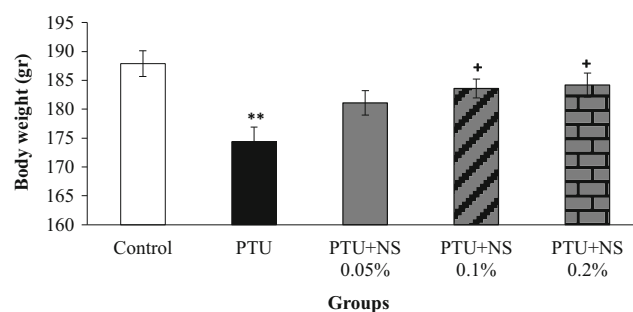
Fig. 1 Comparing the body weight in different groups. Body weight in PTU group was significantly less than the control group. Although the body weight of animals in PTU + NS 0.05% group N. sativa did not significantly change, animals treated by 0.1% and 0.2% W/V N. sativa had a significantly more body weight compared to PTU group. Data are presented as mean ± SEM. **$P < 0.01$ compared to control group, +$P < 0.05$ compared to PTU group

Fig. 2 Light microscopic images of the apoptotic cells (*arrows*) among normal pyramidal cells in coronal sections of CA1, CA3 and DG regions of rat hippocampus in all groups. Apoptotic cells that are present in the area have shrinkage and brown nuclei with irregular cytoplasm
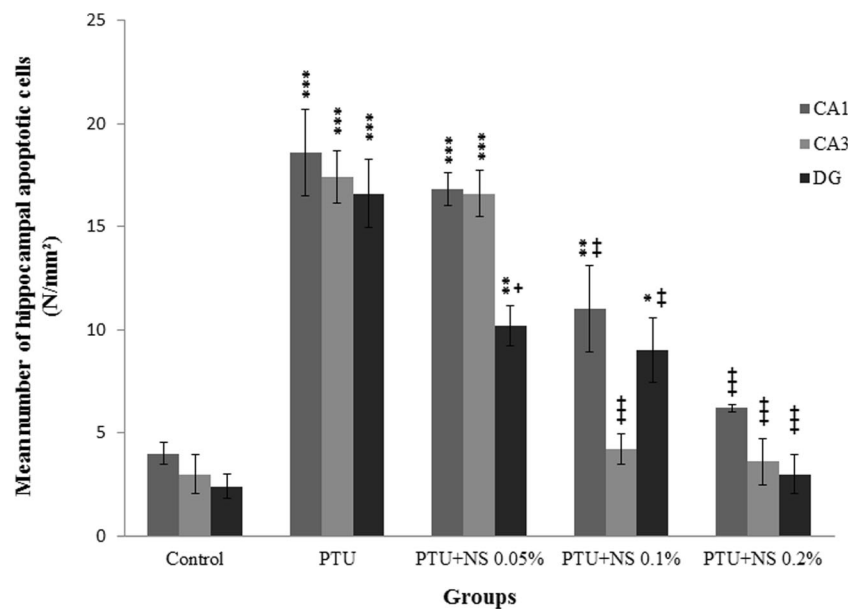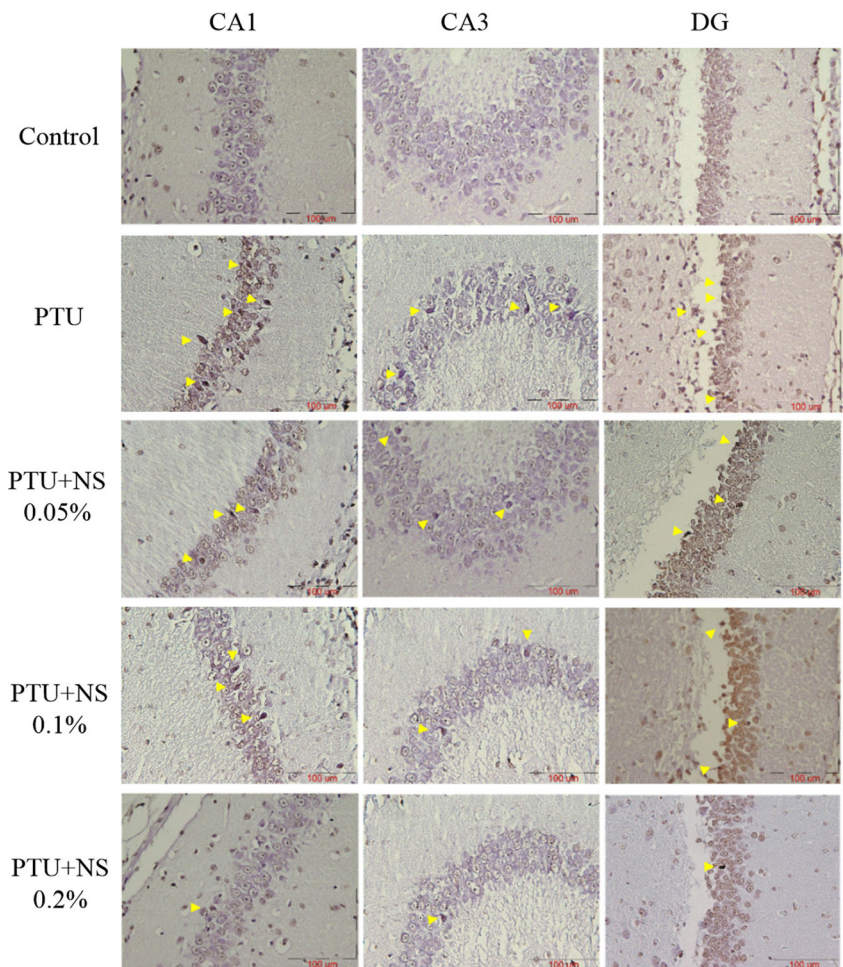
Fig. 3 Comparison of apoptotic cell number per unit area (N/mm^2) in CA1, CA3 and DG areas of the hippocampus between groups. Apoptotic cells in sub- regions of the hippocampus in PTU group were significantly more than the control group. Administration of *N. sativa* extract at concentrations of 0.05% *W/V* significantly reduced the number of apoptotic cells in DG area. Treatment by 0.1% and 0.2% *W/V N. sativa*, showed a significant reduction in the number of apoptotic cells in all regions of the hippocampus. Data are presented as mean ± SEM. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ compared to control group. +$P < 0.05$, ++ $P < 0.01$ and +++ $P < 0.001$ compared to PTU group

Furthermore, the administration of *N. sativa* extract at the concentration of 0.2% *W/V* noticeably reduced the mean number of apoptotic cells in all regions of hippocampus compared to PTU group (*P* < 0.001; Fig. 3).

### The effect of *Nigella sativa* on production of dark neurons

Our findings revealed that hypothyroidism induced by PTU, led to the appearance of dark neurons at CA1, CA3, and DG of the hippocampus in the all tested groups (Fig. 4) however, they were significantly more abundant in the PTU group compared to control group (*P* = 0.001; Fig. 5). Although treatment by *N. sativa* extract at concentration of 0.05% *W/V* didn't significantly change the number of dark neurons in sub-regions of the hippocampus compared to PTU group, administration of 0.1% as well as 0.2% *W/V N. sativa* remarkably declined the mean number of dark neurons per unit area in all regions of hippocampus in comparison with PTU group (*P* < 0.01 and *P* < 0.001; respectively, Fig. 5).

The effect of *Nigella sativa* on numerical density and total number of dark neurons in the hippocampus

The results of this study demonstrated that the mean number of dark neurons per unit area (N/mm^3) in right hippocampus was 607.6 ± 13 in control group and 1428.4 ± 238 in PTU group. Hypothyroidism induced by PTU significantly enhanced the density of dark neurons in the hippocampus (*P* = 0.005; Fig. 6a). Subsequently, the total number of dark neurons in the hippocampus of hypothyroid rats in PTU group significantly increased compared to the control group (*P* = 0.006; Fig. 6b). Administration of 0.05% *W/V Nigella sativa* extract did not change the density of dark neurons which induced by hypothyroidism in the hippocampus (954.6 ± 169; *P* = 0.2). Furthermore, it did not change the total number of dark neurons in the hippocampus of this rats (*P* = 0.2; Fig. 6b). However, administration of 0.1% and 0.2% *W/V* of *N. sativa* extract significantly prevented production of dark neurons as well as total number of these cells in the hippocampus

Fig. 4 The light microscopic appearance of toluidine blue-stained dark neurons at coronal sections of CA1, CA2 and DG of the hippocampus in different groups. Arrows show dark neurons among normal pyramidal cells
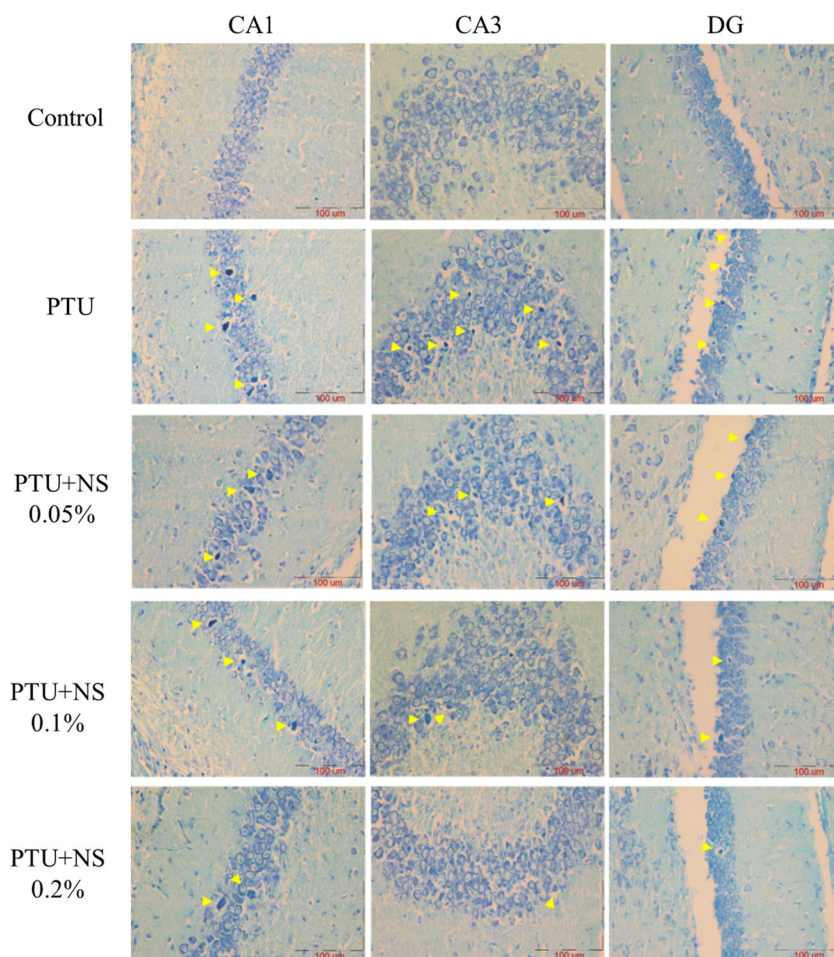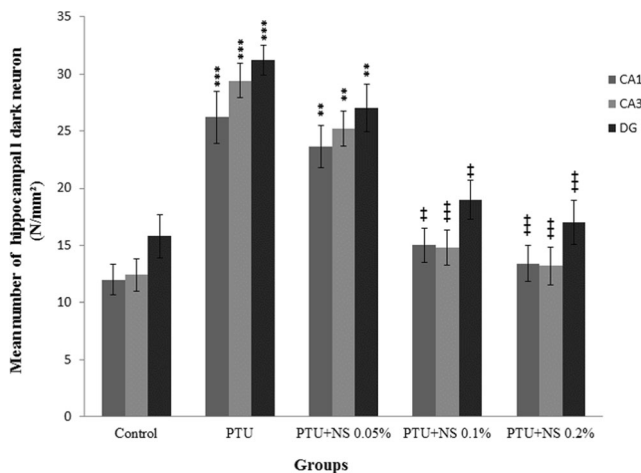
Fig. 5 Comparing the number of dark neurons per unit area (N/mm²) in CA1, CA3 and DG areas of the hippocampus between groups. PTU-induced hypothyroidism significantly enhanced the production of dark neurons in different areas of the hippocampus in rats. Administration of 0.1% and 0.2% W/V N. sativa significantly reduced the production of dark neurons in different areas of the hippocampus. Data are presented as mean ± SEM. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ compared to control group. ++$P < 0.01$ and +++$P < 0.001$ compared to PTU group

of hypothyroid rats and reduced the mean number of dark neurons to 743.6 ± 93.4 and 676.4 ± 80.3, respectively ($P = 0.02$ and $P = 0.01$;, Fig 6a, b).

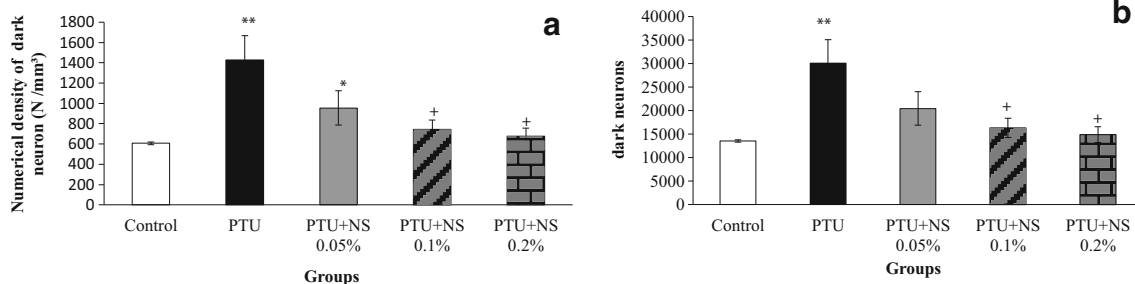### The effect of *Nigella sativa* on the hippocampal volume

According to the Cavalieri method, the mean hippocampal volume for the control group was 22.31 ± 0.15 mm³ and PTU- induced hypothyroidism significantly decreased the volume of the hippocampus to 21.12 ± 0.05 mm³ (P<0.001; Fig. 7). Administration of N. sativa extract at concentrations of 0.1% and 0.2% W/V noticeably attenuated this effect of hypothyroidism on hippocampus compared to the PTU group (P<0.001; Fig. 7). While treatment of the animals by 0.05% W/V N. sativa extract didn't has any significant effect on the

volume of the hippocampus in comparison with PTU group ($P = 0.1$). The coefficient error (CE) was estimated for the hippocampus in different groups (Table 2).

Discussion

This study evaluated the effect of *Nigella sativa* extract during the neonatal and juvenile period on hippocampal volume, the number of dark neurons and apoptotic cells in the hippocampus of the hypothyroidism rats. Our results showed that hypothyroidism induced by 0.005% PTU causes neuronal damage in different regions of the hippocampus, which leads to a reduction of hippocampal volume in the rats. Meanwhile, administration of different concentration of N. sativa hydroalcoholic extract dose-dependently attenuated hypothyroidism- induced hippocampal neuronal damage.

Previous studies showed that hypothyroidism during the neonatal and juvenile period can cause irreparable damage to developing nervous system includes mental retardation, learning, and memory impairment (Ford and Cramer 1977; Hosseini et al. 2012; Legrand 1984). It is documented that thyroid hormones deficiency either during development or at maturity reduces neural proliferation and can lead to increased cell death in hippocampus and cerebellum (Cooke et al. 2014; Sinha et al. 2009). Sinha et al. (2009) showed that TH deficiency is associated with the death of granule neurons within the developing cerebellum and their results indicated that this hypothyroidism-induced neuronal loss involved in an apoptotic dependence signaling. Madeira et al. (1991) demonstrated that hypothyroidism regardless of when the disorder began results in an elimination of the total number of granule cells due to both deficient cell acquisition and neuronal death in the dentate gyrus. In the other study, they found that hypothyroidism induces a reduction in the volume of the pyramidal cell layer in CA3 and in the CA1 region (Madeira et al. 1992). In line with our results, Huang et al. (2005) investigated the effects of hypothyroidism on apoptosis and the

Fig. 6 The effect of *Nigella sativa* extract on the numerical density and a total number of dark neurons at hippocampus in hypothyroid rats. Hypothyroidism induced by PTU significantly increased density (a) and total number (b) of dark neurons in the right hippocampus of rats. *Nigella sativa* at concentration of 0.05% W/V significantly reduced the numerical density of dark neurons (a) whereas; it did not change the total number (b)

of these cells in the hippocampus. Administration of *Nigella sativa* at the concentrations of 0.1% and 0.2% W/V considerably decreased both numerical density and a total number of dark neurons in the hippocampus. Data are presented as mean ± SEM. *$P < 0.05$ and **$P < 0.01$ compared to control group. +$P < 0.05$ compared to PTU group
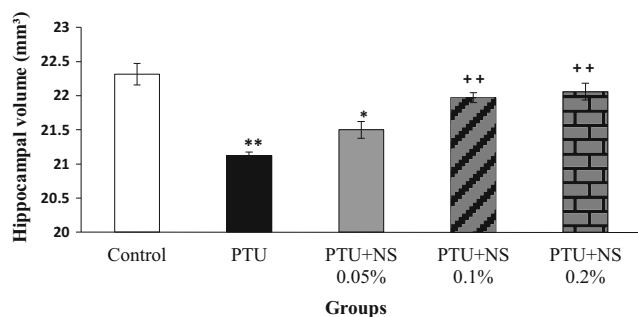
Fig. 7 The effect of *Nigella sativa* extract on the hippocampal volume in hypothyroid rats. PTU- induced hypothyroidism significantly reduced the volume of the right hippocampus. Treatment by 0.05% *W/V N. sativa* extract did not change the volume of the hippocampus. Administration of *Nigella sativa* at the concentrations of 0.1% and 0.2% *W/V* significantly increased the hippocampal volume in rats. Data are presented as mean ± SEM. *$P < 0.05$ and **$P < 0.01$ compared to control group. ++ $P < 0.01$ compared to PTU group

expression of Bax and Bcl-2 gene in the developing rat hippocampus neurons and reported that the thyroid hormones significantly prevents apoptosis of hippocampus neurons. Furthermore, Cooke et al. (2014) showed that hypothyroidism in adult patients causes a significant reduction in the volume of the right hippocampus and this could explain some of the memory deficits that have been observed in those with hypothyroidism.

Although previous studies have been not determined the exact mechanism of hypothyroid damaging effects on the nervous system, some studies reported that the increased nitric oxide (NO) level in the hippocampus of hypothyroidism rats may cause learning and memory impairment during the neonatal period (Cernak et al. 2001; Hosseini et al. 2010). It has been suggested that the NO synthase (NOS) activity enhancement due to TH deficiency in the hippocampus may be associated with an increased level of lipid peroxidation in the brain tissues (Cano-Europa et al. 2008). Furthermore, numerous studies have shown that hypothyroidism induces oxidative stress due to reactive oxygen species (ROS) production (Konukoglu et al. 2002; Yilmaz et al. 2003) and the role of ROS in affecting neuronal death is well-documented (Klein

and Ackerman 2003). Therefore, oxidative stress can be considered as one of the possible causes of neurological disorders and memory impairment induced by hypothyroidism (Beheshti et al. 2017; Farrokhi et al. 2014; McEwen 1999; Wang and Michaelis 2010).

The seeds of *Nigella sativa,* a religion-based remedy that have been prized for their healing properties since ancient times, express many pharmacological actions such as analgesic (Ali and Blunden 2003), anti-tumoral (Musa et al. 2004) and antioxidant (Erkan et al. 2008; Hamdy and Taha 2009) as well as neuroprotective effects (Sahak et al. 2013). Evidence suggests that antioxidants have beneficial effects in preventing memory disorders (Bhutada et al. 2011) and play an important role in the evolution of the nervous system by protecting nerve cells against oxidative damage (Mitchell et al. 1999). Sahak et al. (2013) regarding the antioxidant and neuroprotective properties of *N. sativa* showed that the use of *N. sativa* oil have beneficial effects on memory and learn abilities in rats. Moreover, previous studies demonstrated the neuroprotective and antioxidant effects of *N. sativa* on hippocampal neural damage and memory impairment in seizure models (Ezz et al. 2011; Seghatoleslam et al. 2016; Vafaee et al. 2015). It is shown that thymoquinone (TQ), the most abundant component of *N. sativa* seeds, is the principle responsible for many of the seed's beneficial effects (Gali-Muhtasib et al. 2006). *N. sativa* and its derived TQ have useful effects in neurodegeneration after chronic toluene exposure on the hippocampus in rats (Kanter 2008). Moreover, recent studies have demonstrated that *N. sativa* extract and TQ have neuroprotective effects and prevent cell death in hippocampal structures following experimental cerebral ischemia (Al-Majed et al. 2006; Hobbenaghi et al. 2014).

Consistent with previous studies the results of the current study showed that the two concentrations (0.1% and 0.2% *W/V*) of the hydrochloric extract of *N. sativa* significantly decreased the total number and numerical density of dark neurons, as well as apoptotic cell production in subdivisions of the hippocampus at hypothyroidism- induced rats. Additionally, these concentrations of *N. sativa* extract attenuated the effect of hypothyroidism on the volume reduction of the hippocampus in this animal.

Table 2 The mean right hippocampal volume, the total number of dark neurons in the right hippocampus, and coefficient of error (CE) in the different groups

Group	Treatment	Hippocampal volume	Total number of dark neurons	C.E
1	Control	22.31 ± 0.15	13,552.7 ± 237.1	0.026
2	0.005% PTU	21.12 ± 0.05**	30,127.3 ± 4978.3**	0.028
3	PTU+ 0.05% *W/V NS*	21.5 ± 0.12*	20,457.9 ± 3533.2	0.027
4	PTU+ 0.1% *W/V NS*	21.97 ± 0.07++	16,323.9 ± 2014.5+	0.027
5	PTU+ 0.2% *W/V NS*	22.058 ± 0.12++	14,894.4 ± 1702.9+	0.026

* $P < 0.05$ and ** $P < 0.01$ compared to control group
+ $P < 0.05$ and ++ $P < 0.01$ compared to PTU group

Beheshti et al. (2017) showed that the *N. sativa* extract has protective effects on hypothyroidism-associated learning and memory impairment during neonatal and juvenile growth in rats. They indicated that the *N. sativa* extract attenuated malondialdehyde (MDA) as a marker of lipid peroxidation while, improved thiol contents in both the cortical and hippocampal tissues of hypothyroid rats. (Beheshti et al. 2017). Then, they suggested protection against oxidative stress in brain tissue may be involved in the beneficial effects of *N. sativa* in brain function in hypothyroidism. Other reports also confirmed the protective effects of *N. sativa* and its main component "TQ" against brain tissues oxidative damage (Ahlatci et al. 2014; Erşahin et al. 2011; Hassanein and El-Amir 2017) and considered that as a possible mechanism(s) for learning and memory improving effects (Hosseini et al. 2015; Vafaee et al. 2015). Additionally, the beneficial effects of *N. sativa* and TQ in the nervous system have been attributed to protection against NO overproduction (Abdel-Zaher et al. 2011; Ezz et al. 2011; Hamdy and Taha 2009; Ozugurlu et al. 2005). It has also been reported that both *N. sativa* and TQ reduce NO and peroxynitrite (ONOO(−)) levels as well as NOS enzyme activity in the brain and protect the brain tissues from radiation-induced nitrosative stress (Ahlatci et al. 2014). On the other hand, an increased level of NO level in the hippocampus of hypothyroidism is considerable (Cernak et al. 2001; Hosseini et al. 2010). Considering this evidence, protection against hippocampal tissues oxidative damage as an explanation for neuroprotective effects of the plant's extract which was seen in the present study might be suggested and these beneficial effects of the plant may be due, in part at least, to inhibition of NOS and protection against overproduction of nitric oxide nevertheless, it needs to be more investigated in the future.

Overall, the results would seem to suggest that hydro-alcoholic extract of the *Nigella sativa* has a strength to prevent dark neuron and apoptotic cell production in the hippocampal regions, as its neuroprotective effects against TH deficiency, and it might be suggested to be used as an effective medication in reducing the adverse effects of hypothyroidism. However, future studies on the current topic are recommended.

Compliance with ethical standards

Conflict of interest The authors have no conflict of interest to declare.

References

Abdel-Zaher AO, Abdel-Rahman MS, Elwasei FM (2011) Protective effect of *Nigella sativa* oil against tramadol-induced tolerance and dependence in mice: role of nitric oxide and oxidative stress. Neurotoxicology 32(6):725–733. doi:10.1016/j.neuro.2011.08.001

Ahlatci A, Kuzhan A, Taysi S, Demirtas OC, Alkis HE, Tarakcioglu M, Demirci A, Caglayan D, Saricicek E, Cinar K (2014) Radiation-modifying abilities of *Nigella sativa* and thymoquinone on radiation-induced nitrosative stress in the brain tissue. Phytomedicine 15 21(5):740–744. doi:10.1016/j.Phymed.2013.10.023

Ali BH, Blunden G (2003) Pharmacological and toxicological properties of *Nigella sativa*. Phytother Res 17(4):299–305. doi:10.1002/ptr.1309

Al-Majed AA, Al-Omar FA, Nagi MN (2006) Neuroprotective effects of thymoquinone against transient forebrain ischemia in the rat hippocampus. Eur J Pharmacol 543(1–3):40–47. doi:10.1016/j.ejphar.2006.05.046

Ataei ML, Ebrahimzadeh-Bideskan AR (2014) The effects of nano-silver and garlic administration during pregnancy on neuron apoptosis in rat offspring hippocampus. Iran J Basic Med Sci 17(6):411–418

Bagheri-Abassi F, Alavi H, Mohammadipour A, Motejaded F, Ebrahimzadeh- Bideskan A (2015) The effect of silver nanoparticles on apoptosis and dark neuron production in rat hippocampus. Iran J Basic Med Sci 18(7):644–648.

Beheshti F, Hosseini M, Shafei MN, Soukhtanloo M, Ghasemi S, Vafaee F et al (2017) The effects of *Nigella sativa* extract on hypothyroidism-associated learning and memory impairment during neonatal and juvenile growth in rats. Nutr Neurosci 20(1):49–59. doi:10.1179/1476830514Y.0000000144

Bhutada P, Mundhada Y, Bansod K, Tawari S, Patil S, Dixit P et al (2011) Protection of cholinergic and antioxidant system contributes to the effect of berberine ameliorating memory dysfunction in rat model of streptozotocin-induced diabetes. Behav Brain Res 220(1):30–41. doi:10.1016/j.bbr.2011.01.022

Bratton SB, MacFarlane M, Cain K, Cohen GM (2000) Protein complexes activate distinct caspase cascades in death receptor and stress-induced apoptosis. Exp Cell Res 256(1):27–33. doi:10.1006/excr.2000.4835

Cano-Europa E, Perez-Severiano F, Vergara P, Ortiz-Butron R, Rios C, Segovia J et al (2008) Hypothyroidism induces selective oxidative stress in amygdala and hippocampus of rat. Metab Brain Dis 23(3):275–287. doi:10.1007/s11011-008-9099-0

Cernak I, Wang Z, Jiang J, Bian X, Savic J (2001) Cognitive deficits following blast injury-induced neurotrauma: possible involvement of nitric oxide. Brain Inj 15(7):593–612. doi:10.1080/02699050010009559

Chareyron LJ, Banta Lavenex P, Amaral DG, Lavenex P (2011) Stereological analysis of the rat and monkey amygdala. J Comp Neurol 519(16):3218–3239. doi:10.1002/cne.22677

Cooke GE, Mullally S, Correia N, O'Mara SM, Gibney J (2014) Hippocampal volume is decreased in adults with hypothyroidism. Thyroid 24(3):433–440. doi:10.1089/thy.2013.0058

Cragg BG (1970) Synapses and membranous bodies in experimental hypothyroidism. Brain Res 18(2):297–307. doi:10.1016/0006-8993(70)90330-6

Dityatev A, Dityateva G, Sytnyk V, Delling M, Toni N, Nikonenko I et al (2004) Polysialylated neural cell adhesion molecule promotes re-modeling and formation of hippocampal synapses. J Neurosci 24(42):9372–9382. doi:10.1523/JNEUROSCI.1702-04.2004

Erkan N, Ayranci G, Ayranci E (2008) Antioxidant activities of rosemary (*Rosmarinus officinalis* L.) extract, blackseed (*Nigella sativa* L.) essential oil, carnosic acid, rosmarinic acid and sesamol. Food Chem 110(1):76–82. doi:10.1016/j.foodchem.2008.01.058

Erşahin M, Toklu HZ, Akakin D, Yuksel M, Yeğen BC, Sener G (2011) The effects of *Nigella sativa* against oxidative injury in a rat model

of subarachnoid hemorrhage. Acta Neurochir 153(2):333–341. doi: 10.1007/s00701-010-0853-9

Ezz HSA, Khadrawy YA, Noor NA (2011) The neuroprotective effect of curcumin and *Nigella sativa* oil against oxidative stress in the pilocarpine model of epilepsy: a comparison with valproate. Neurochem Res 36(11):2195–2204. doi:10.1007/s11064-011-0544-9

Farrokhi E, Hosseini M, Beheshti F, Vafaee F, Hadjzadeh MA, Dastgheib SS (2014) Brain tissues oxidative damage as a possible mechanism of deleterious effects of Propylthiouracil- induced hypothyroidism on learning and memory in neonatal and juvenile growth in rats. Basic Clin Neurosci 5(4):285–294

Ford DH, Cramer EB. 1977 Developing nervous system in relation to thyroid hormones. Thyroid hormones and brain development: Raven Press, New York. p. 1–18.

Gali-Muhtasib H, El-Najjar N, Schneider-Stock R (2006) The medicinal potential of black seed (*Nigella sativa*) and its components. In: Mahmud THK, Arjumand a, editors. Advances in Phytomedicine 2:133–153. doi:10.1016/S1572-557X(05)02008-8

Gong J, Liu W, Dong J, Wang Y, Xu H, Wei W et al (2010) Developmental iodine efficiency and hypothyroidism impair neural development in rat hippocampus: involvement of doublecortin and NCAM-180. BMC Neurosci 11(1):1. doi:10.1186/1471-2202-11-50

Gupta VK, Sharma SK (2006) Plants as natural antioxidants. Nat Prod Radiance 5(4):326–334 http://hdl.handle.net/123456789/7962

Hamdy NM, Taha RA (2009) Effects of *Nigella sativa* oil and thymoquinone on oxidative stress and neuropathy in streptozotocin-induced diabetic rats. Pharmacology 84(3):127–134. doi:10.1159/000234466

Hassanein KM, El-Amir YO (2017) Protective effects of thymoquinone and avenanthramides on titanium dioxide nanoparticles induced toxicity in Sprague-Dawley rats. Pathol Res Pract 213(1):13–22. doi: 10.1016/j.prp.2016.08.002

Hobbenaghi R, Javanbakht J, Sadeghzadeh S, Kheradmand D, Abdi F, Jaberi M et al (2014) Neuroprotective effects of *Nigella sativa* extract on cell death in hippocampal neurons following experimental global cerebral ischemia-reperfusion injury in rats. J Neurol Sci 337(1):74–79. doi:10.1016/j.jns.2013.11.019

Hosseini M, Dastghaib SS, Rafatpanah H, Hadjzadeh MA-R, Nahrevanian H, Farrokhi I (2010) Nitric oxide contributes to learning and memory deficits observed in hypothyroid rats during neonatal and juvenile growth. Clinics 65:1175–1181. doi:10.1590/S1807-59322010001100021

Hosseini M, Shafei MN, Safari V, Taiarani Z, Kafami Ladani M, Sadeghian R (2012) The effects of olibanum administered to methimazole-treated dams during lactation on learning and memory of offspring rats. Nat Prod Res 26(16):1544–1548. doi:10.1080/14786419.2011.566223

Hosseini M, Mohammadpour T, Karami R, Rajaei Z, Reza Sadeghnia H, Soukhtanloo M (2015) Effects of the hydro-alcoholic extract of *Nigella sativa* on scopolamine-induced spatial memory impairment in rats and its possible mechanism. Chin J Integr Med 21(6):438–444. doi:10.1007/s11655-014-1742-5

Hosseinzadeh H, Parvardeh S, Asl MN, Sadeghnia HR, Ziaee T (2007) Effect of thymoquinone and *Nigella sativa* seeds oil on lipid peroxidation level during global cerebral ischemia-reperfusion injury in rat hippocampus. Phytomedicine 14(9):621–627 http://phypha.ir/ppj/article-1-374-en.html

Huang XW, Zhao ZY, Ji C (2005) Effects of hypothyroidism on apoptosis and the expression of Bcl-2 and Bax gene in the neonatal rat hippocampus neurons. Zhonghua Er Ke Za Zhi 43(1):48–52

Islam MH, Ahmad IZ, Salman MT (2015) Neuroprotective effects of *Nigella sativa* extracts during germination on central nervous system. Pharmacogn Mag 11:182. doi:10.4103/0973-1296.157729

Jalali M, Tehranipour M (2013) Effect of alcoholic extract of *Nigella sativa* seed on alpha motor neurons density of spinal cord following sciatic

nerve compression in rats. J Gorgan Univ med Sci 15(4): 29-34. http://goums.Ac.Ir/journal/article-1-866-en.Html. (article in Farsi)

Jannini EA, Ulisse S, D'Armiento M (1995) Thyroid hormone and male gonadal function. Endocr Rev 16(4):443–459. doi:10.1210/edrv-16-4-443

Kanter M (2008) *Nigella sativa* And derived thymoquinone prevents hippocampal neurodegeneration after chronic toluene exposure in rats. Neurochem Res 33(3):579–588. doi:10.1007/s11064-007-9481-z

Khan AA, Ramli NB, Ismail ZM (2015) The effects of progesterone on hypoxic ischemic injuries in the Cornu Ammonis (CA) region of the hippocampus of neonatal rats. Int J Morphol 33(3):962–970. doi:10.4067/S0717-95022015000300025

Klein JA, Ackerman SL (2003) Oxidative stress, cell cycle, and neurodegeneration. JCI Insight 111(6):785–793. doi:10.1172/JCI200318182

Konukoglu D, Ercan M, Hatemi H (2002) Plasma viscosity in female patients with hypothyroidism: effects of oxidative stress and cholesterol. Clin Hemorheol Microcirc 27(2):107–113

Legrand J (1984) Effects of thyroid hormones on central nervous system development. Neurobehav Toxicol:331–363

Madeira MD, Cadete-Leite A, Andrade JP, Paula-Barbosa MM (1991) Effects of hypothyroidism upon the granular layer of the dentate gyrus in male and female adult rats: a morphometric study. J Comp Neurol 314(1):171–186. doi:10.1002/cne.903140116

Madeira MD, Sousa N, Lima-Andrade MT, Calheiros F, Cadete-Leite A, Paula-Barbosa MM (1992) Selective vulnerability of the hippocampal pyramidal neurons to hypothyroidism in male and female rats. J Comp Neurol 322(4):501–518. doi:10.1002/cne.903220405

Mancini A, Di Segni C, Raimondo S, Olivieri G, Silvestrini A, Meucci E et al (2016) Thyroid hormones, oxidative stress, and inflammation. Mediat Inflamm 2016:1–12. doi:10.1155/2016/6757154

McEwen BS (1999) Stress and hippocampal plasticity. Annu Rev Neurosci 22:105–122. doi:10.1146/annurev.neuro.22.1.105

Mitchell JJ, Paiva M, Heaton MB (1999) Vitamin E and beta-carotene protect against ethanol combined with ischemia in an embryonic rat hippocampal culture model of fetal alcohol syndrome. Neurosci Lett 263(2–3):189–192. doi:10.1016/S0304-3940(99)00144-5

Morreale de Escobar G, Obregon MJ, Escobar del Rey F (2004) Role of thyroid hormone during early brain development. Eur J Endocrinol 151:25–37. doi:10.1530/eje.0.151U025

Musa D, Dilsiz N, Gumushan H, Ulakoglu G, Bitiren M (2004) Antitumor activity of an ethanol extract of *Nigella sativa* seeds. Biologia, Bratislava 59(6):735–740

Namavar MR, Raminfard S, Jahromi ZV, Azari H (2012) Effects of high-fat diet on the numerical density and number of neuronal cells and the volume of the mouse hypothalamus: a stereological study. Anat Cell Biol 45(3):178–184. doi:10.5115/acb.2012.45.3.178

Ozugurlu F, Sahin S, Idiz N, Akyol O, Ilhan A, Yigitoglu R, Isik B (2005) The effect of *Nigella sativa* oil against experimental allergic encephalomyelitis via nitric oxide and other oxidative stress parameters. Cell Mol Biol (Noisy-le-grand) 51(3):337–342

Pourzaki M, Homayoun M, Sadeghi S, Seghatoleslam M, Hosseini M, Ebrahimzadeh Bideskan A (2017) Preventive effect of *Coriandrum sativum* on neuronal damages in pentylentetrazole-induced seizure in rats. Avicenna J Phytomed 7(2):116–128. doi:10.22038/ajp.2016.7757

Rajabzadeh AA, Bideskan AR, Haghir H, Fazel AR (2011) Morphometrical study of polysialylated neural cell adhesion molecule positive cells in rat pups hippocampus following induction of seizure during pregnancy. Iran Biomed J 15(4):157–163

Sahak MK, Mohamed AM, Hashim NH, Hasan Adli DS (2013) Nigella Sativa oil enhances the spatial working memory performance of rats on a radial arm maze. Evid Based Complement Alternat Med 2013: 5. doi:10.1155/2013/180598

Salem ML (2005) Immunomodulatory and therapeutic properties of the Nigella Sativa L. seed. Int. Immunopharmacology 5(13):1749–1770. doi:10.1016/j.intimp.2005.06.008

Sedaghat R, Roghani M, Khalili M (2014) Neuroprotective effect of thymoquinone, the nigella sativa bioactive compound, in 6-hydroxydopamine-induced hemiparkinsonian rat model. Iran J Pharm Res 13(1):227–234

Seghatoleslam M, Jalali M, Nikravesh MR, Alamdari DH, Hosseini M, Fazel A (2013) Intravenous administration of human umbilical cord blood-mononuclear cells dose-dependently relieve neurologic deficits in rat intracerebral hemorrhage model. Ann Anat 195(1):39–49. doi:10.1016/j.aanat.2012.05.002

Seghatoleslam M, Alipour F, Shafieian R, Hassanzadeh Z, Edalatmanesh MA, Sadeghnia HR et al (2016) The effects of Nigella Sativa on neural damage after pentylenetetrazole induced seizures in rats. J Tradit Complement Med 6(3):262–268. doi:10.1016/j.jtcme.2015.06.003

Sharif SH, Elmahdi BM, Mohammed AMA, Mohammed AH (2012) The effects of Nigella Sativa L. ethanolic extract on thyroid function in normal and alloxaninduced diabetic rats. Thyroid res Pract 9(2):48. doi:10.4103/0973-0354.96044

Shibutani M, Woo G-H, Fujimoto H, Saegusa Y, Takahashi M, Inoue K et al (2009) Assessment of developmental effects of hypothyroidism in rats from in utero and lactation exposure to anti-thyroid agents. Reprod Toxicol 28(3):297–307. doi:10.1016/j.reprotox.2009.04.011

Shin M-S, Ko I-G, Kim S-E, Kim B-K, Kim T-S, Lee S-H et al (2013) Treadmill exercise ameliorates symptoms of methimazole-induced hypothyroidism through enhancing neurogenesis and suppressing apoptosis in the hippocampus of rat pups. Int J Dev Neurosci 31(3):214–223. doi:10.1016/j.ijdevneu.2013.01.003

Sinha RA, Pathak A, Kumar A, Tiwari M, Shrivastava A, Godbole MM (2009) Enhanced neuronal loss under perinatal hypothyroidism involves impaired neurotrophic signaling and increased proteolysis of p75(NTR). Mol Cell Neurosci 40(3):354–364. doi:10.1016/j.mcn.2008.12.001

Vafaee F, Hosseini M, Hassanzadeh Z, Edalatmanesh MA, Sadeghnia HR, Seghatoleslam M et al (2015) The effects of Nigella Sativa hydro-alcoholic extract on memory and brain tissues oxidative damage after repeated seizures in rats. Iran J Pharm Res 14(2):547–557

Wagner MS, Wajner SM, Maia AL (2008) The role of thyroid hormone in testicular development and function. J Endocrinol 199(3):351–365. doi:10.1677/JOE-08-0218

Wang X, Michaelis EK (2010) Selective neuronal vulnerability to oxidative stress in the brain. Front Aging Neurosci 2:12. doi:10.3389/fnagi.2010.00012

West MJ (1993) New stereological methods for counting neurons. Neurobiol Aging 14(4):275–285

West MJ, Gundersen H (1990) Unbiased stereological estimation of the number of neurons in the human hippocampus. J Comp Neurol 296(1):1–22. doi:10.1002/cne.902960102

Yilmaz S, Ozan S, Benzer F, Canatan H (2003) Oxidative damage and antioxidant enzyme activities in experimental hypothyroidism. Cell Biochem Funct 21(4):325–330. doi:10.1002/cbf.1031

Zsombok A, Tóth Z, Gallyas F (2005) Basophilia, acidophilia and argyrophilia of "dark"(compacted) neurons during their formation, recovery or death in an otherwise undamaged environment. J Neurosci Meth 142(1):145–152. doi:10.1016/j.jneumeth.2004.08.005