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Vitamin C prevents hypothyroidism associated neuronal damage in the hippocampus of neonatal and juvenile rats: A stereological study

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ABSTRACT

Hypothyroidism causes an imbalance in antioxidant and pro-oxidants criteria in the brain and enhances the concentration of reactive oxygen species (ROS), and neuronal damage has been observed following an excessive ROS. The main purpose of this study was to examine the preventive effect of vitamin C on hypothyroidism associated neuronal damage in the hippocampus of neonatal and juvenile rats.

Pregnant rats after delivery of their pups were randomly divided into four groups and treated with (1) normal drinking water as a control group, (2) Propylthiouracil (PTU) 0.005% added to drinking water, (3-, 4) PTU + Vit C 10 mg/ kg and PTU + Vit C 100 mg/ kg to drinking water.

Treatment was carried out during rat's lactation period until to the postnatal day (PND) 60. To assess the histological and stereological changes that occur in this study, brains of 5 male pups were extracted.

The number of dark neurons and apoptotic cells in the hippocampal sub-regions of PTU group was significantly greater than the control group's hippocampal sub-regions. In addition, hypothyroidism induced a reduction in the hippocampal volume and increased the numerical density and the total amount of dark neurons. The vitamin C only dose of 100 mg/kg significantly reduced the number of dark neurons and apoptotic cells ($P < 0.01$) and considerably weakened the influence of hypothyroidism on the volume reduction of the hippocampus ($P < 0.05$).

The current study suggested that vitamin C administration has a possibility to prevent hippocampal neuronal damage caused by neonatal and juvenile hypothyroidism in rats.

1. Introduction

It is well established that thyroid hormones (THs) are vital for regulating mammal's growth and development, and play an important role in the nervous system's development and normal brain function (Cano-Europa et al., 2008; Gao et al., 2014; Sahoo and Roy, 2012). In addition, it has been reported that thyroid dysfunction in adults, is linked to neurological and behavioral abnormalities (Cano-Europa et al., 2008; Gao et al., 2014; Sahoo and Roy, 2012; Wagner et al., 2009; Wajner et al., 2009). Many studies have proven that thyroid disorders such as hypothyroidism not only result in intellectual disability, cognitive and memory impairments (Pan et al., 2013) but also reduce the granular and pyramidal cells in Dentate Gyrus (DG) and Cornu Ammonis (CA1) of hippocampus respectively (Madeira et al., 1992, 1991). The hippocampal formation appears to be particularly sensitive to the thyroid hormones (McEwen and Milner, 2007) and based on the previous studies, the neuronal damage, which presents by generation of dark neurons (Asiaei et al., 2017; Madeira et al., 1991) and apoptotic cells (Asiaei et al., 2017; Sinha et al., 2009), occurs following hypothyroidism.

Dark neurons that are experimentally and clinically reported in the neuropathology may be observed under particular conditions such as head injury, stroke, seizure, and pathological metabolic status like hypoglycemia (Kherani and Auer, 2008). These electron-dense nuclei and cytoplasm cells (Cattani et al., 2013; Rahaman et al., 2001) can be recognized by their hyperbasophily, hyperargyrophily, and high-electron dense properties in histological sections (Sadeghi et al., 2013; Seghatoleslam et al., 2016). The fate of dark neurons, about to either die or recover depends on the severity of brain insults (Ishida et al., 2004) and it is still unclear whether or not dark neurons represents a

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distinct type of neuronal cell death (Yang et al., 2008). However, apoptotic cells which are characterized by specific morphological characteristics including cytoplasmic shrinkage and condensed nuclear chromatin and segregation, and can be easily recognized using TUNEL [Terminal deoxynucleotidyl transferase (TdT) dUTP nick end labeling] assay (Pourzaki et al., 2017) are definitely a form of neuronal cell death.

Metabolically, the brain is an active organ with a maximal oxygen intake and huge amounts of polyunsaturated fatty acids that produce a huge amount of ROS (Pan et al., 2013). ROS are considered as neurotoxic molecules, which utilize their deleterious effects through essential macromolecules oxidation such as enzymes (Pan et al., 2013). There is a relation between excessive ROS production in the brain and decreased performance in cognitive function (Pan et al., 2013). They also affect synaptic plasticity with accompanying neuronal damage in animals (Zaidi and Banu, 2004). Clinical and experimental studies showed a very high production of ROS and oxidative stress in hypothyroidism (Abedelhaffez and Hassan, 2013; Babu et al., 2011) and it has been indicated that hypothyroidism increases oxidative damage in cerebral tissue and this damage cannot be prevented despite increases in antioxidant system activity (Kumari et al., 2011; Pasupathi and Latha, 2008).

Vitamin C (ascorbic acid) a water-soluble vitamin, which is required as a co-factor for many enzymes, involves in the synthesis or modulation of some components in the nervous system. (Katsuki, 1996). It is a well-known antioxidant molecule in the brain and many researchers have suggested that it acts as a ROS scavenger especially on the newly formed neurons (Ebrahimzadeh-Bideskan et al., 2016; Harrison and May, 2009). In addition, vitamin C may decrease reaction between toxins and the critical biomolecule, as well as creates a genomic protection through suppression of intracellular ROS, and inhibit apoptosis (Khordad et al., 2013). The concentration of vitamin C in body fluids and tissues depends on intestinal absorption, cellular transport, and excretion and it has been documented that the leukocytes, eye, adrenals, pituitary and brain preserve the highest level of vitamin C (Jacob and Sotoudeh, 2002). It seems that this high level of vitamin C may play a main role in the protection of brain against oxidative stress. Several in vitro studies have shown that adding ascorbic acid to cultured cells, brain slices and brain microsomes prevents lipid peroxidation induced by various oxidizing agents (Harrison and May, 2009; Iwata et al., 2014). In addition, some in vivo studies especially those dealing with seizure and cerebral ischemia defined that vitamin C has a neuroprotective effect on neuronal degeneration (Mack et al., 2006; Naseer et al., 2011).

Regarding previous studies and based on the antioxidant and neuroprotective properties of vitamin C, this study was designed to determine whether the administration of vitamin C in hypothyroid rats prevents neuronal damage and ameliorate the volume reduction of the hippocampus during neonatal and juvenile growth.

2. Material and methods

2.1. Study design and experimental groups

Twenty adult Wistar female rats (200–250 g body weight, 10–12 weeks old) after mating and performing vaginal smear, pregnant rats were housed in separate cages at 22 ± 2 °C, at a room with 12:12-h light/dark cycle and access to both food and water. All the experiments involving the rats handling and related procedures were performed according to Mashhad University of Medical Sciences, Ethical Committee Acts. Animals were then randomly assigned to four groups (n = 5):

1. Control group was administered normal drinking water.
2. Propylthiouracil (PTU) group was administered drinking water containing 0.005% PTU (Sigma Co., St. Louis, USA) to induce the hypothyroidism (Bhanja and Chainy, 2010).
3. PTU + Vit C 10 group was treated with 0.005% PTU-supplemented drinking water and 10 mg/kg vitamin C (Vijayprasad et al., 2014).
4. PTU + Vit C 100 group received 0.005% PTU-supplemented drinking water and 100 mg/kg vitamin C (Li et al., 2008).

To calculate the first desired volume of drinking water, the mean volume of consumed water by each pregnant rat was measured for 3 days and then the supplements were freshly prepared and appropriately added to the foil-covered water bottles. All treatments were sustained during lactation period and were extended until postnatal day (PND) 60. Due to the variable nature of female data caused by hormonal fluctuations related to the menstrual cycle, after the lactation period, the male offspring separated and the volume of consumed water was measured for them and an appropriate amount of materials was dissolved in their drinking water.

At the end of treatment period, in each group, five male pups were randomly weighed and a radioimmunoassay kit (DIAsource T4-RIA-CT Kit, Belgium; Cat. No. KIP1641) measured their serum T4 level to detect the hypothyroidism.

2.2. Tissue preparation and staining

At the final day of the experiment (PND 60), selected animals deeply anesthetized with a ketamine/xylazine mixture (90/10 mg/kg i.p.) (Routh et al., 2009) and then, transcardially perfused with cold saline followed by fixative solution (10% buffered formalin). After that, their brains removed and immersed in the same fixative solution for 48 h. A common tissue processing and paraffin embedding were applied to brain samples. Subsequently, by a rotatory microtome (Leitz 1512, Germany) serial coronal brain sections (5 μm thickness) were obtained. The boundary of hippocampus [Cornu Ammonis (CA) including CA1, CA2, CA3 and DG] was defined in accordance to the stereotaxic atlas (Paxinos and Watson, 2013). Then for the TUNEL assay, an average of 10 equally distant slices including the hippocampus from every block of brains was chosen and mounted on the Poly-L-lysine coated slides. Since there is no specific method available for dark neurons detection and a common way for detecting of these cells is based on their increased stain ability with cationic dyes (such as Toluidine blue or Cresol violet) as well as silver staining method (Zsombok et al., 2005), the rest of slides were stained with 0.1% Toluidine blue. A light microscope equipped with a digital high-resolution camera (BX51, Olympus, Japan), was used to capture images from brain slices at magnification 40×, 20 x and 4 x objective lenses (UPlan FI, Japan).

2.3. Apoptotic cell detection

In order to apoptotic cell detection, TUNEL immunohistochemical technique was done using TUNEL Kit (Roche, Germany; Cat. No. 11684817910) which detects DNA fragmentations in the apoptotic cell nuclei and presents them as dark brown cells (Asiaei et al., 2017). To perform this technique, tissue sections were deparaffinized and rehydration rinsed in 0.1 M PBS (Phosphate-buffered saline) for 10 min and then incubated with 20 μg/ml proteinase K (Roche, Germany; Cat. No. 03115836001) at ambient temperature for 20 min, and then for inactivation of endogenous peroxidase, they were treated with 3% H2O2 in methanol for 10 min. After washing at PBS, samples were incubated in the labeling reaction mixture containing terminal deoxynucleotidyl transferase and the deoxynucleotide mixture overnight in the dark at 4 °C. The next day, all sections after rinsing in PBS incubated with horseradish peroxidase (1:500) at room temperature for 30 min. Subsequently, the sections were washed at PBS for 3 min and then DAB (3, 3- Diaminobenzidine) (Sigma, USA; CAS No. 91–95-2) solution (30 mg diaminobenzidine and 200 μl H2O2/100 ml PBS) was applied in the dark at room temperature for 15 min. After a thorough wash with running water, all the sections were stained with hematoxylin and