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The Protective effect of P7C3 against DNA and neuron damage in rat pups with congenital hypothyroidism

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ABSTRACT

Congenital hypothyroidism (CH) is defined as congenital thyroid hormone deficiency. The aim of this study was to examine the DNA and neuron damage in rat pups with CH and to evaluate the beneficial effects of 3.6-Dibromo-α-[(phenylamino) methyl]-9H-carbazole-9-ethanol (P7C3). Rat pups were assigned to four groups as Group 1: CH, Group 2: CH treated with P7C3, Group 3: CH treated with P7C3 and L-thyroxine, and Group 4: control group. Plasma 8-(OH)DG and neuron-specific enolase (NSE) concentrations were determined in all groups. For histopathological examinations haematoxylin-eosin staining was applied. Increased NSE concentrations were found in the CH group compared to the control group. The 8-(OH)DG concentrations were found to be higher in Group 2 and Group 3 than in the control group. Neuronal degenerations localized in the hippocampus and brain cortex were found in histopathological examinations in Group 1. The distribution of neuronal degeneration was less in Group 2 and Group 3 than Group 1 and lesser in Group 3 than in Group 2. DNA damage might have a role in CH pathogenesis. P7C3 compounds have a protective effect in CH.

1. Introduction

Congenital hypothyroidism (CH) is defined as congenital thyroid hormone deficiency characterized by low concentrations of thyroxine (T4) [1]. It occurs in about 1 in 3,000–4,000 newborns [2]. However, only 25%–33% of the overall birth population can be screened for CH in many developing countries, so CH cases are generally detected clinically [3]. Causes of thyroid hormone insufficiency during gestation include a lack of dietary iodine, thyroid dysgenesis and dysmorphogenesis [4]. Thyroid hormones are essential for neurogenesis, neuronal migration, myelination and synaptogenesis, which are essential for proper neurodevelopment. Pathologically low concentrations of thyroid hormones during critical stages of neurological development result in many structural defects such as mental retardation, deaf-mutism, spastic dysplegia and extrapyramidal rigidity [5,6].

8-hydroxy-2′ -deoxyguanosine [8-(OH)DG] is formed as a result of oxidative damage to DNA by reactive oxygen and nitrogen species [7]. The interaction of HO with the nucleobases of the DNA strand, such as guanine, leads to the formation 8-(OH)DG [8]. Several studies have examined 8-(OH)DG in different neurological diseases, but there is still insufficient data about the changes of 8-(OH)DG concentrations in CH. Neuron-Specific Enolase (NSE) is a glycolytic isoenzyme which is expressed by mature neurons and cells of neuronal origin [9]. The levels of NSE have been used to evaluate the implications of brain damage in experimental studies on rats [10,11]. In addition, NSE has been suggested as a biomarker to define neuronal alterations in chronic disease including Creutzfeldt-Jakob disease, Alzheimer's disease and diabetic neuropathy [12–14]. To the best of our knowledge, there has been no study on the changes of NSE levels in CH.

Successful treatments for CH are mainly dependent on several factors such as early diagnosis, and determination of the optimal dose and timing of thyroid hormone replacement. Lower and higher doses of L-thyroxine may lead to a poorer outcome and symptoms of excessive thyroid hormone, respectively [15,16]. P7C3 is a novel neuroprotective and pro-neurogenic small molecule. P7C3 fosters the survival of neurons and protects the hippocampus neural progenitor cells against apoptotic cell death [17–19]. Recent studies have reported that it may be potentially useful for the treatment of neurological disorders including traumatic brain injury, stroke, amyotrophic lateral sclerosis, peripheral nerve cell death, Parkinson's disease, Alzheimer's disease, Huntington's disease and neuropsychiatric diseases[20–28].

Therefore, in the present study, a rat model of congenital hypothyroidism generated by exposure to methimazole (MMI) was used to examine histopathological findings and the changes of plasma 8-(OH)

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DG and NSE concentrations in rat pups. In addition, the effects were evaluated of P7C3 supplementation and L-thyroxine on histopathological findings and the concentrations of plasma 8-(OH)DG and NSE. This study can be considered to provide an important contribution to the existing literature regarding CH.

2. Methods

2.1. Animal exposure

Approval for the study was granted by the Local Ethics Board of Cumhuriyet University (decision no.65202830-050.04.04-30). Male and female Wistar rats were mated in the laboratory, which was maintained at a temperature of 22 °C–24 °C and 55% humidity with a 12-hr light/l2-hr dark cycle (light between 6:00 am and 6:00 pm). The appearance of vaginal plugs was considered as gestation day 0 (GD 0). A colony of 8 pregnant rats was equally and randomly divided into 4 groups. Six rat pups were randomly selected and euthanasia was performed at postnatal 21 (P21) days after parturition in each group. Detailed descriptions of the experimental procedures are given below. All procedures were performed according to the guidelines stated in the National Institute of Health Guide for the Care and Use of Laboratory Animals.

Group 1 (n:6): MMI (0.025% wt/vol) was administered daily in drinking water to the pregnant rats from GD9 to P21 to generate pups with congenital hypothyroidism.

Group 2 (n:6): MMI (0.025% wt/vol) was administered daily in drinking water to the pregnant rats from GD9 to P21. From P1 to P21 the rat pups were administered P7C3 (Tocris Bioscience, Bristol, UK, CAS no: 301353-96-8) at a dose of 10mg/kg/day by oral gavage.

Group 3 (n:6): MMI (0.025% wt/vol) was administered daily in drinking water to the pregnant rats from GD9 to P21. From P1 to P21 the rat pups were administered P7C3 at a dose of 10mg/kg/day by oral gavage, and levothyroxine dissolved in 0.9% Sodium chloride (NaCl) at a dose of 7.5 μg/day/kg was injected subcutaneously.

Group 4: (n:6) The pregnant rats were fed ad libitum and normal tap water without MMI from GD0 to P21. The rat pups were fed breast milk from their lactating mothers and were kept in the same cage as their own dams until P21.

2.2. Blood samples

Blood samples were collected from all rat pups into pediatric lavender top tubes (Becton Dickinson, UK). After centrifugation at 4 °C for 15 min at 3500 rpm, the plasma was aliquoted and immediately frozen at −80 °C (Wisecryo, Korea). To determine the efficacy of MMI administration, the plasma TSH and fT4 concentrations of the pregnant rats were determined in the second week of gestation.

2.3. Measurement of plasma NSE, fT4, TSH and 8-(OH)DG concentrations

The quantitative sandwich ELISA technique was used for the determination of plasma NSE (MyBioSource), 8-(OH)DG (Enzo Life Sciences, PRC) and Thyroid-Stimulating Hormone (TSH) (Elabscience Biotechnology Inc.). Free T4 (fT4) levels were measured using immunoassays (Cobas, e601,Roche Diagnostics). The tests were performed according to the manufacturer's recommendations. The intra-assay and inter-assay CV values of the kits were < 10% and < 12%, respectively. The main limitation of plasma NSE measurement is that it is present in erythrocytes at particularly high concentrations. Therefore, no samples were analyzed with hemolysis.

2.4. Histopathological examination

Tissue samples collected from the hippocampus and other parts of the brain cortex were trimmed and processed routinely and embedded in paraffin wax. Sections of 6μm thickness were cut from the paraffin blocks. The sections were stained according to the haematoxylin-eosin (H&E) staining procedure and evaluated under a light microscope and illuminated using a camera attachment (Euromex digital microscope). For scoring of histopathological findings in all brain cortex and hippocampus, degenerated and necrotic neuronal cells were counted totally at 10 High Power Fields in 400x magnification (10 HPFs) in all groups.

2.5. Statistical analysis

The normal distribution of data was evaluated with the Shapiro-Wilk normality test. Numerical variables were shown as mean ± SD The ANOVA tests and Greenhouse-Geisser correction were used to compare the NSE and 8-(OH)DG concentrations between groups, and the Tukey's multiple comparisons test was used in all binary comparisons. ANOVA and the Bonferroni's post hoctests were used to comparison of histopathological scores between groups. Data analyses were performed with GraphPad Prism version 7.00 for Windows software (GraphPad Software, La Jolla California USA, www.graphpad.com).

3. Results

3.1. Biochemical findings

Plasma fT4 concentrations were lower in the groups where the pregnant rats were administered methimazole only or simultaneously methimazole + P7C3 compared to the control group ($p < 0.001$). The plasma fT4 and TSH concentrations of the pregnant rats are shown in Table 1. Increased plasma mean NSE concentrations were found in the methimazole-treated group compared to the control group. Decreased mean concentrations of NSE were found in the methimazole + P7C3 group compared to the methimazole-treated group. The rate of mean NSE concentration reduction was much greater in the L-thyroxine + P7C3-treated group than in the P7C3 only group. Multiple comparisons of NSE concentrations between the groups are shown in Graph 1. 8-(OH)DG concentrations were found to be higher in methimazole-treated groups than in the control group. No difference was determined between the methimazole-treated group, the methimazole + P7C3-treated group and the P7C3 + methimazole + L-thyroxine-treated group in terms of 8-(OH)DG concentrations ($p > 0.05$). Multiple comparisons of 8-(OH)DG concentrations between the groups are shown in Graph 2. The mean fT4, TSH, NSE and 8-(OH)DG concentrations of the rat pups on day P21 are shown in Table 2.

3.2. Histopathological findings

Neuronal degenerations including karyopyknosis and cytoplasmic shrinkage in different parts of the brain cortex and hippocampus [Cornu ammonis 1 (CA1, CA2, CA3) and dentate gyrus (DG)] especially in

Table 1
Plasma TSH and fT4 concentrations of pregnant rats in the second week of gestation.

Groups	Pregnant rats	TSH (mIU/mL)	fT4 (ng/dL)
Group 1	PR1	12.36 ± 0.17	0.78
	PR2	10.05 ± 0.46	0.32
Group 2	PR1	6.68 ± 0.62	0.35
	PR2	9.03 ± 0.33	0.31
Group 3	PR1	10.21 ± 0.76	0.72
	PR2	8.39 ± 0.48	1.48
Group 4 (Control)	PR1	2.28 ± 0.14	3.26
	PR2	2.62 ± 0.21	3.52

PR: pregnant rat, fT4: free thyroxin, TSH: Thyroid stimulating hormone. Results are expressed as mean ± SD with 95% confidence intervals.