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Protective effects of GM-CSF in experimental neonatal hypothyroidism

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ABSTRACT

Hypothyroidism induced by methimazole (MMI), has a negative impact on the postnatal development. Neonatal Granulocyte Macrophage-Colony Stimulating Factor [GM-CSF; 50 μg/kg, intramuscular injection at postnatal day (PND) 17] had been tested to ameliorate the effects of MMI [0.05%, (weight per volume; *w*/*v*), intraperitoneal injection at PND 15]-induced hypothyroidism in Wistar rats. The hypothyroid conditions due to the administration of MMI produced inhibitory effects on neonatal serum thyroxine (T4), 3,5,3′-triiodothyronine (T3), neutrophil count in bone marrow and blood, cerebellar glutathione (GSH) and acetylcholinesterase (AchE), although it induced stimulatory actions on serum thyrotropin (TSH), growth hormone (GH), insulin growth factor-II (IGF-II), tumor necrosis factor alpha (TNF-α), and cerebellar malondialdehyde (MDA) at PND 19. The treatment with GM-CSF could reverse the depressing and stimulating effects of MMI on these markers except for cerebellar AchE where its enhancement was non-significant ($P > 0.05$) at tested PND. Thus, neonatal GM-CSF may be responsible for suppressing autoimmune responses and preventing hypothyroidism.

1. Introduction

Thyroid hormones (THs) could regulate the development and critical biochemical functions in developing brain [1–3]. MMI (anti-thyroid drug) could inhibit THs synthesis because it could interfere with the conversion of iodide (I^-) to iodine (I°) and consequently, the iodination of tyrosyl groups [4]. Neonatal rats which received MMI in their mother's milk rendered hypothyroid [5]. This deficiency could delay the growth and result in an irreversible impairment, maldevelopment, physical retardation and neural dysfunctions [6]. Hypothyroidism associated also with disturbed cytokines [7], AchE activity [8], and the balance between the generation of reactive oxygen species (ROS) and antioxidants in most developing brain regions [9]. Oxidative stress as a result of hypothyroidism could lead to cellular ionic imbalance, signal transduction, and enzyme activity modifications in mammalian central nervous system (CNS) [10]. This variation may predispose structures to oxidative stress-related neurodegenerative disorders.

THs dysfunctions caused by MMI exposure have been shown to suppress the hematopoiesis and immune system [7,11]. GM-CSF is produced by normal human thyrocytes and regulates the cell development and function [12,13]. It has a specific activity on progenitor cells of neutrophils and its administration in experimental animals could increase the neutrophils in bone marrow and blood [14,15]. In addition, it has been applied for treatment of different disorders like thyroid dysfunctions [16,17], embryo teratogenesis in diabetic pregnant mice [18], healing process and extra-hepatic systemic metastases [19,20]. Since MMI severely affect the neonatal development, the aim of this study was to examine the ability of GM-CSF in treating the MMI-induced hypothyroidism in neonatal rats through measurement of serum THs, TSH, GH, IGH-II and TNF-α. Moreover, the current study purposed to detect the changes in the activity of AchE and prooxidant (lipid peroxidation; LPO)/antioxidant (GSH; main brain antioxidant) markers in neonatal cerebellum. In this regard, cerebellum was used as a model system because this region is highly sensitive to any stress (TH disturbance) and its development occurs in postnatal rats [21]. This developmental stage reflects the time period between the 3rd trimester of gestation to the 2nd postnatal year in the human [22].

2. Materials and methods

2.1. Chemicals

MMI was purchased from Sigma-Aldrich, St. Louis, MO, USA while GM-CSF was obtained from Novartis Pharma, Switzerland. T4, T3, TSH, GH, IGF-II and TNF-α kits were purchased from Calbiotech INC

Abbreviations: AchE, acetylcholinesterase; AchRs, acetylcholine receptors; ATCI, acetylthiocholine iodide; CNS, central nervous system; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; GH, growth hormone; GM-CSF, Granulocyte Macrophage-Colony Stimulating Factor; GSH, glutathione; HPTA, hypothalamic–pituitary–thyroid axis; I^-, iodide; I°, iodine; IGF-II, insulin growth factor—II; LPO, lipid peroxidation; MDA, malondialdehyde; MMI, methimazole; PND, postnatal day; ROS, reactive oxygen species; T3, 3,5,3′-triiodothyronine; T4, thyroxine; TBA, thiobarbituric acid; TCA, trichloroacetic acid; TNF-α, tumor necrosis factor alpha; TPO, thyroperoxidase; Tregs, T-regulatory cells; TRs, thyroid receptors; TSH, Thyrotropin.

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(CBI), USA. All other reagents were of the purest grades commercially available.

2.2. Animals and treatments

The experimental animals used in this study were male white albino rats (*Rattus norvegicus* (Wistar strain); 40 pups aged 15 PND). The rats were obtained from the National Institute of Ophthalmology, Giza, Egypt. They were fed a standard rodent pellet diet manufactured by an Egyptian company producing oil and soap as well as some vegetables as a source of vitamins. Tap water was provided and the rats were allowed to drink *ad libitum*. The rats were exposed to constant daily 12 h light: 12 h darkness each (lights on at 06:00 h) and 50 ± 5% relative humidity. All the procedures used for the experimental animals were in accordance with the guidelines and the recommendations of the Canadian Council on Animal Care (CCAC) [23]. All efforts were made to minimize the number of animals used and their suffering.

The experimental animals were divided into four groups of 10 rats each. Single injection of 0.05% (weight per volume; *w*/*v*) MMI [24] was intraperitoneal at PND 15 while recombinant human GM-CSF (50 μg/kg; Leucomax flacon, 150 mg) was intramuscular at PND 17. Groups 1 and 2 received 0.5 ml of sterile saline but Groups 3 and 4 received 0.5 ml of MMI at PND 15. After 48 h (PND 17), Groups 2 and 4 received recombinant GM-CSF [25]. Two days later (PND 19), animals were euthanized, blood samples were collected and centrifuged at 10,000 rpm. Neonatal cerebellum was homogenized in 0.25 M cold sucrose by using a Teflon homogenizer (Glas-Col, Terre Haute, USA) and kept at −70 °C.

2.3. Quantification of circulating and storage neutrophils

Total blood leukocytes were counted using the Digicell 500 cell counter (Contraves AG, Switzerland). Blood smears were prepared and stained with Wright stain, and a 100–200 cell differential count was performed. Absolute neutrophil counts were determined by the multiplication of the nucleated cell count by the percentage of neutrophils in the differentials. Neutrophil bone marrow pools were determined [26]. Briefly, postnatal femurs were aseptically removed and the contents were flushed into a known quantity of hank's buffer salt solution (HBSS; Gibco Laboratories, Grand Island, NY). Total cell counts were performed, and a 500-cell differential count was obtained on Wright-stained cytospin preparations.

2.4. RIA examination

The concentrations of serum T4 [27], T3 [28], TSH [29], GH [30], IGF-II [31] and TNF-α [32] were estimated quantitatively by RIA in Diabetic Endocrine Metabolic Pediatric Unit, Center for Social and Preventive Medicine, New Children Hospital, Faculty of Medicine, Cairo University, Egypt.

2.5. Developmental and biochemical markers in neonatal cerebellum

2.5.1. Determination of acetylcholinesterases activity

The AchE activity was assessed by standard spectrophotometric Ellman's method. Acetylthiocholine iodide (ATCI) was used as an appropriate substrate and 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) was used as a chromogen [33]. The activity was expressed as μmole of ATCI hydrolyzed/min/mg of protein. All measurements were done in duplicate. The data were normalized to the amount of protein measured by the Lowry method, using the Bio-Rad DC protein assay and bovine serum albumin as the standard.

2.5.2. Glutathione (GSH) concentration

The method was based on the development of a yellow color when DTNB was added to compounds containing sulfhydryl groups [34]. Supernatants in phosphate buffer (500 μL) were added to 3 mL of 4% sulfosalicylic acid. The mixture were centrifuged at 1600 ×*g* for 15 min. Supernatants (500 μL) were taken and added to Ellman's reagent. The absorbance was measured at 412 nm after 10 min. The GSH concentration was expressed as nmol/100 mg tissue.

2.5.3. Malondialdehyde (MDA) level

The MDA concentrations, index of LPO, were determined spectrophotometrically [35]. Briefly, supernatant was mixed with 1 mL of 5% trichloroacetic acid (TCA) and centrifuged at 2500 ×*g* for 10 min. An amount of 1 mL of thiobarbituric acid (TBA) reagent (0.67%) was added to 500 μL of the supernatant and heated at 90 °C for 15 min. The mixture was then cooled and measured for absorbance at 532 nm. The MDA values were calculated by using 1,1′,3,3′-tetraethoxypropane as standard and expressed as nmoles MDA/100 mg/h.

2.6. Statistical analysis

SPSS (version 20) statistical program (SPSS Inc., Chicago, IL) was used to carry out a one-way analysis of variance (ANOVA) on our data. When significant differences by ANOVA were detected, analysis of differences between the means of the treated and control groups were performed by using Dunnett's t test.

3. Results

3.1. Effect of GM-CSF on MMI-induced decrease in circulating and storage neutrophils

The treatment with GM-CSF could prevent neutropenia caused by MMI at PND 19 (Fig. 1). Compared to the control group, MMI did decrease circulating (68.1%) and storage (55.1%) neutrophils significantly

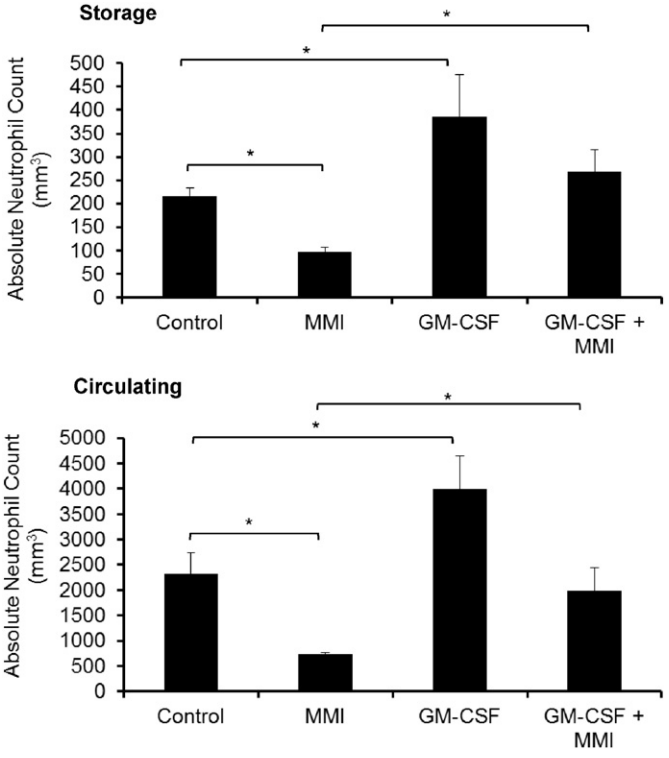

Fig. 1. Effect of recombinant GM-CSF on MMI-induced hypothyroidism: Neutrophil count in bone marrow (storage) and blood (circulating) at PND 19. The values are means ± SEM (n = 6). MMI is methimazole [0.05% (*w*/*v*), intraperitoneal injection at PND 15] and GM-CSF is Granulocyte Macrophage-Colony Stimulating Factor (50 μg/kg, intramuscular injection at PND 17). * denotes to significant difference at $P < 0.05$.

(P < 0.05), while GM-CSF alone increased them (41.7 and 43.5%, respectively; P < 0.05). Treatment with GM-CSF could restore the levels of decreased circulating and storage neutrophils (62.8% and 30.5%, respectively; P < 0.05) after MMI treatment.

3.2. Effect of GM-CSF on MMI-induced alterations in neonatal serum markers at PND 19

MMI induced a depression in T3 and T4 levels (66.6 and 50.4%, respectively; P < 0.001; Fig. 2). GM-CSF did not show any effect on the levels of THs but could reverse the depressive effect of MMI (57.1 and 40.1%, respectively; P < 0.001). Compared to the control, hypothyroid group showed an increase in TSH, GH, IGF-II and TNF-α levels (56.5, 47, 82.3, 38.6%, respectively; P < 0.001), while their levels were increased (34.5, 38.8, 63.7 and 26.6%, respectively; P < 0.001) in hypothyroid treated group. Similarly to the THs, GM-CSF alone did not show any effects on the levels of these markers (Figs. 3 and 4).

3.3. Effect of GM-CSF on MMI-induced disruption to the developmental and biochemical markers in neonatal cerebellum at PND 19

The hypothyroid group showed an increase in the oxidative stress through decreased GSH (44.9%, P < 0.001) and increased MDA levels (58.2%, P < 0.001) which are a marker for lipid peroxidation (Fig. 5). Treatment with GM-CSF could in both cases inhibit (45.7 and 25.4%, respectively; P < 0.001) the effect of MMI. Similarly to the decreased THs and GSH levels, the activity of AchE was decreased (30.4%; P < 0.05) in hypothyroid group compared to control groups. However, the activity of AchE in MMI group was not significantly (P > 0.05) enhanced by GM-CSF treatment.

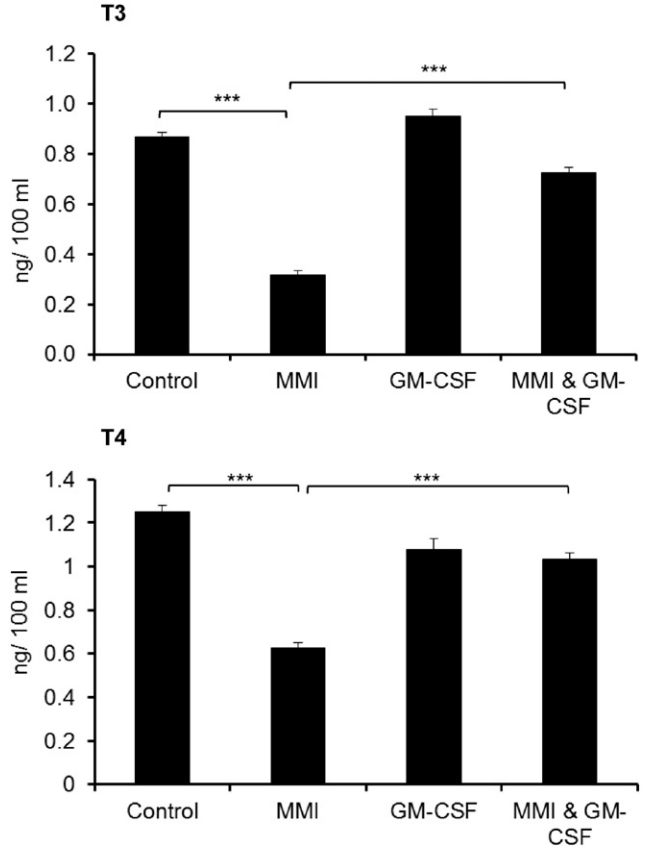

Fig. 2. Effect of neonatal recombinant GM-CSF on MMI-induced hypothyroidism: Serum T3 and T4 levels at PND 19. The values are means ± SEM (n = 6). MMI is methimazole [0.05% (*w*/*v*), intraperitoneal injection at PND 15] and GM-CSF is Granulocyte Macrophage-Colony Stimulating Factor (50 μg/kg, intramuscular injection at PND 17). *** denotes to significant difference at P < 0.001.

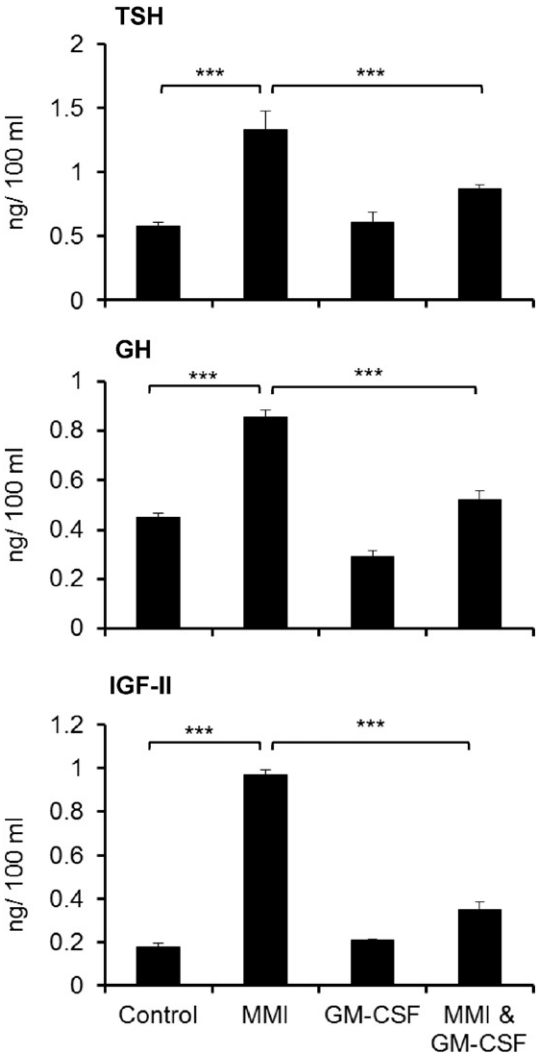

Fig. 3. Effect of recombinant GM-CSF on MMI-induced hypothyroidism: Serum TSH, GH and IGF-II levels at PND 19. The values are means ± SEM (n = 6). MMI is methimazole [0.05% (*w*/*v*), intraperitoneal injection at PND 15] and GM-CSF is Granulocyte Macrophage-Colony Stimulating Factor (50 μg/kg, intramuscular injection at PND 17). *** denotes to significant difference at P < 0.001.

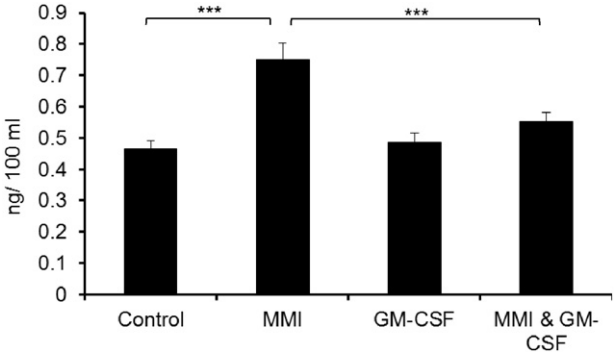

Fig. 4. Effect of recombinant GM-CSF on MMI-induced hypothyroidism: Serum TNF-α level at PND 19. The values are means ± SEM (n = 6). MMI is methimazole [0.05% (*w*/*v*), intraperitoneal injection at PND 15] and GM-CSF is Granulocyte Macrophage-Colony Stimulating Factor (50 μg/kg, intramuscular injection at PND 17). *** denotes to significant difference at P < 0.001.

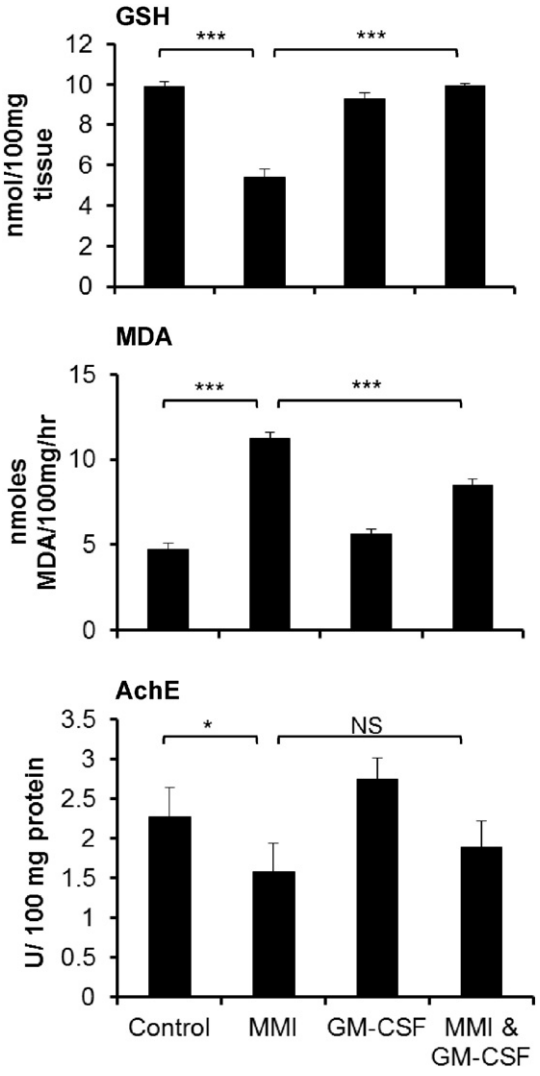

Fig. 5. Effect of recombinant GM-CSF on MMI-induced hypothyroidism: Cerebellar MDA, GSH and AchE levels at PND 19. The values are means ± SEM (n = 6). MMI is methimazole [0.05% (*w*/*v*), intraperitoneal injection at PND 15] and GM-CSF is Granulocyte Macrophage-Colony Stimulating Factor (50 μg/kg, intramuscular injection at PND 17). * denotes to significant difference at $P < 0.05$ while *** denotes to $P < 0.001$. NS denotes to non-significant.

4. Discussion

Previous studies reported the immaturity of immune responses in perinatal and neonatal development periods and recommended using different recombinant cytokines including GM-CSF for immune stimulation [36,37]. Furthermore, hypothyroidism correlated with immune dysfunction and decreased count of immune cells in spleen and bone marrow [38]. MMI, as one of the anti-thyroid drugs, could reduce the myeloid colony growth leading to agranulocytosis [39,40]. GM-CSF had been able to increase the hematopoiesis of neutrophils and increase their number in blood [14,15]. Therefore, it was interesting to study the effect of GM-CSF on experimentally induced hypothyroidism in rat neonates through assessment of THs, TSH, GH, IGF, TNF-α and cerebellar oxidative stress & AchE activity. THs, TSH and IGF, GH, TNFα and AchE activity were found to be vital in the development [41–44].

MMI has been applied in this study to develop hypothyroidism in neonatal rats. It was found to decrease the serum THs levels and neutrophil count in both bone marrow and blood, while the levels of serum TSH, GH, IGF-II and TNF-α were elevated at examined PND compared to control group. MMI could induce its action either by reducing the binding of T3 to nuclear thyroid receptors (TRs) [45], inhibiting thyroperoxidase (TPO) activity (which normally oxidizes anion I^- to I° during T4 synthesis in the thyroid gland), blocking the organification of thyroglobulin or delaying THs synthesis [46]. Similar to previous findings, the fall in THs associated with elevated TSH, GH and IGF-II [47,48]. The elevated TNF-α level indicated an increase in proinflammatory cytokine release in hypothyroidism [49].

Treatment with GM-CSF with a single dose (50 μg/kg) was applied in this study to avoid the neonatal oxidative stress [50]. This treatment indicated an antagonistic effect on MMI-induced hypothyroidism which was related to reduced neutrophil counts and THs but increased TSH, GH, IGF-II and TNF-α levels. Similarly, this cytokine was able to modulate teratogen-induced effects by activation of immune cells and their migration into the utero and placental compartments [51]. GM-CSF was able to suppress experimental autoimmune thyroiditis/thyroid dysfunction [17] by blocking the TPO antibodies [52], activating the regulatory T cells [17] or activating neutrophils, macrophages, monocytes and eosinophils [53]. This indicates that the decreased level of TNF-α after GM-CSF treatment was essential mechanism of action against hypothyroidism. In addition, TNF-α was found to increase apoptotic mechanisms in embryo [54]. The immunoprotective action of GM-CSF could ensure immune system homeostasis and decrease the vulnerability to stress-induced disease (7). Generally, the neonatal treatment with GM-CSF could improve the defense mechanisms *via* regulating hematopoiesis, THs, growth factors and TNF-α.

The increased neonatal cerebellar MDA level in the current hypothyroid state was associated with elevated TNF-α level and reduced cerebellar GSH & AchE levels at PND 19 in comparison with their corresponding control. Thus, the augmented oxidative stress was related to increase of TNF-α level and decreased AchE activity [55]. These results supported the hypothesis that decreased TH may be a relevant predictor for long-lasting developmental neurotoxicity [56]. This could be a result of impaired synapse formation and dysregulation of neurotransmitters [57]. Indeed, hypothyroidism was found associated with several brain disorders and neuro-degeneration in postnatal life [58]. The inverse link between TNF-α and THs or AchE which could be responsible for hypothalamic–pituitary–thyroid axis (HPTA) dysfunction had been proved. This could lead, in turn, to the malfunction of developing cerebellum and has deleterious effect on the health of the newborns and adulthood [43]. Thus, the dysfunction in neonatal thyroid–brain axis of the MMI-hypothyroid group might be a main cause of developmental disorders.

Our current findings strongly support the working hypothesis that maintaining the balance between antioxidants (as cerebellar GSH) and ROS (cerebellar MDA) during postnatal period may modulate the neonatal healthy life [43].This protection might be mediated by THs which regulate the growth and brain development [59]. AchE activity was maintained in the brain during the postnatal period, and THs could regulate its activity through their action on nerve growth factor or through their genomic and non-genomic actions [60]. Furthermore, GH and IGF (neurotrophic and neuroprotective factors) axis could regulate its activity during pre- and post-weaning periods [61].

GM-CSF appeared to have a protective effect against MMI-induced oxidative stress and neuro-damage in developing cerebellum. This can be attributed to the suppressing effect of GM-CSF on serum TNF-α and cerebellar MDA related inflammatory response. Also, this treatment could increase the cerebellar GSH and AchE but the latter was not significant at tested day. This can be explained as GM-CSF could modulate the development of the immune system, reduce production of antibodies against the acetylcholine receptors (AchRs) and promote defense against hypothyroidism [62]. Furthermore, GM-CSF was found as essential in dendritic cell survival and differentiation (*in vitro* and *in vivo*) and in the development of neonatal CNS [63,64]. Indeed, the maternal immune stimulation was found to decrease the mRNA expression of TNFα in embryo and protect against the teratogenic insult [65]. Thus, optimal neonatal immune health may be important for protection against events leading to certain neonatal defects. These results support

the view that immune/thyroid–brain interactions might be important in the defense and support the developing cerebellum.

In conclusion, neonatal hypothyroidism might cause pathophysiological and patho-development states which impair HPTA, GH/IGF axis and the development of cerebellum. Also, the deficiency of THs might be directly related to the impairment of the cellular immune system (neutropenia) and enhancement of cerebellar oxidative stress. Therefore, it is necessary to develop new anti-thyroid drugs without causing oxidative stress and cellular damage. Moreover, GM-CSF as a hematopoietic cytokine was an effective treatment against the neonatal hypothyroidism. These protective effects might be either directly or indirectly related to TH action, and depend on the intensity and nature of the dose, experimental duration, developmental period, and type of biological fraction studied. Further research is needed to investigate the molecular mechanisms that regulate GM-CSF production. Also, further studies are required to elucidate the potential associations with human health.

Conflict of interest

The authors declare that no competing financial interests exist.

Acknowledgments

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