Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

Original Research

The effect of Hordeum vulgare on the monoaminergic system modulating neural-thyroid dysfunction in hypothyroid female rats

Lobna F. Wahman[1,2], Marwa M. Abd- Rabo[2], Magda H. M. Youssef [1*], Usama K. Abdel-Hameed[1,3]

[1] Faculty of Medicine, Taibah University, KSA
[2] National Organization for Drug Control and Research (NODCAR), Giza, Egypt
[3] Faculty of Science, Ain -Shams University, Egypt

[*] **Correspondence to:** mhm.youssef@gmail.com

Received January 17, 2019; **Accepted** April 18, 2019; **Published** April 30, 2019

Doi: http://dx.doi.org/10.14715/cmb/2019.65.4.9

Abstract: Thyroid hormones regulate the development and maturation of the brain by maintaining levels of neurotransmitters and their related metabolites. The present work emphasizes the neural dysfunction in the brain caused by hypothyroidism and the potential role of *Hordeum vulgare* (water soluble barley, (B)) in ameliorating these effects. The study was conducted on euothyroid and hypothyroid adult female rats. The induction of hypothyroidism was conducted by oral-administration of neo-mercazole (5.0 $mg.kg^{-1}$) daily for thirty days prior the study and terminated at the end of the study. The groups were assigned as; euthyroid (EU) and hypothyroid (H) groups and other two groups were treated with 100 $mg.kg^{-1}$ water soluble barley; daily for one month and assigned as (EU+B) and (H+B) groups. Compared with EU and EU+B groups, a reduction in fT4, and ERK1/2 levels and elevation in TSH in brain tissue, Moreover, a significant elevation in 8-OH deoxyguanosine and caspase-3 levels, confirmed with increase percentage DNA-damage in the brain and thyroid tissues in hypothyroid control rats. Furthermore, a significant decrease in all monoamines levels in different brain areas and downregulation of dopamine and 5-hydroxytreptamin receptors transcription, with a significant increase in excitatory amino acids and no significant change in the levels inhibitory amino acids were recorded in control hypothyroid group. Treatment of hypothyroid group with *Hordeum vulgare* improved the above-mentioned adverse impact by ameliorating the thyroid hormone levels with depleting the DNA-degradation and elaborating the levels of neurotransmitters with related receptors and amino acids in brain areas. Water soluble *Hordeum vulgare* as a phytonutrient, is safe and efficient agent in ameliorating the neural dysfunction resulting from hypothyroidism status in adult female rats.

Key words: Hypothyroidism; Monoamines; DNA-damage; Apoptosis**;** *Hordeum vulgare* (water soluble barley)**.**

Introduction

Hypothyroidism is a highly prevalent condition that impairs learning and memory and it can also induce delayed skeletal development, cardiovascular diseases, secondary hypertension, the deterioration of human reproductive health and brain dysfunction (1) . The incidence of thyroid disorders is directly proportional to age and is higher among women than men, with rates of 4.98/1000 and 0.88/1000 per year, respectively (2). The thyroid hormones (THs) include T_4 and triiodothyronine T_3, which is the most biologically active form. In plasma, the ratio of T_4 and T_3 is approximately 20:1. Approximately 99% of T_3 results from the peripheral conversion of T_4 by deiodinases (5'-iodinase); thus, T_3 is three-to four-fold more potent than T_4. Pituitary thyroid-stimulating hormone (TSH) stimulates thyroid gland secretion, which is involved in many vital processes throughout the body, such as growth, development, differentiation and metabolism (3).

TSH is synthesized and secreted by the adenohypophysis lobe and exerts its effect by binding to the cognate thyrotropin receptor (TSHR) which is primarily located on the cell surface of the thyroid follicle. The binding of TSH to TSHR on thyroid epithelial cells stimulates the production of thyroglobulin and thyroid peroxidase proteins, which are essential for the synthesis and secretion of THs (4).

THs bind their nuclear receptors (TRs), which are present in many tissues and organs in the human body and hence regulate their functions. The genomic actions of THs is mediated by the binding of T_3 to TRs, which in turn either inhibits or activates gene expression and regulates developmental and metabolic processes by coordinating large gene expression networks (5). Thyroxin-binding globulin (TBG) is responsible for transporting T_3 and T_4 in the circulation. Most T_4 (75%) binds to TBG for transport, whereas the remaining T_4 binds transthyretin (TTR) or albumin or circulates as a free fraction (6).

The development and maturation of the human brain requires THs, which influence a wide range of developmental processes, such as myelination, neuronal and glial cell differentiation, and the synthesis of key enzymes required for neurotransmitter formation. The genes involved in these processes are regulated by THs; thus, hypothyroidism may cause reduced axonal growth and dendritic arborization in the cerebral cortex, visual cortex, auditory cortex, hippocampus, and cerebellum, as well as impaired memory, cognitive function and attentiveness. During the prenatal period hypothyroidism results in irreversible brain damage and mental retarda-

tion (7–9). A previous study (10) provided three lines of evidence describing the roles of TH in brain function. First, T_3 binds TRs located throughout the adult rat brain, which are particularly abundant in the amygdala and hippocampus. Second, the process of 5-deioniation by which T_3 and T_4 are metabolized to inactive iodothyronines has been observed in different adult brain tissues. Third, high THs levels are detected in cortical tissues.

The hippocampus is a part of the limbic system and plays important roles in long-term memory, learning processes and spatial navigation. Sufficient levels of TH during hippocampal development control its structural integrity (11). Hypothyroidism in adult rats reduces the thickness of postsynaptic densities (PSDs). In contrast, brain-derived neurotrophic factor (BDNF) levels are elevated compared with those in control animals. All of these changes are associated with gliosis and neuronal death in the hippocampus (8).

Neurotransmitters are molecules that transmit signals to other neurons to promote communication. These molecules are released from storage vesicles in the presynaptic neuron and bind to receptors on postsynaptic-cells. TH plays a role in neurotransmitter release and hence mood regulation by regulating post-receptor signal transduction and gene expression (9). TH also modulates the levels of several neurotransmitters, such as norepinephrine (NE), epinephrine, 5-HT and DA which are responsible for maintaining a good mental state and preventing depression (7). In the Arab culture, *Hordeum vulgare* or barley syrup is used to relieve depression. Barley is categorized into the spring and winter types, which are considered two-rowed or six-roweddepending on the number of seed rows on each spike. Based on its grain composition, barley is further classified into normal, waxy or high amylose starch type, and contains high levels of β-glucan, proanthocyanidin, protein (12); vitamin E; nicotinic acid; pyridoxine; folic acid; essential amino acids, such as tryptophan and phenylalanine; neutral amino acids (LNAA), such as the three branched chain aromatic amino acids leucine, isoleucine, and valine (13). Barley grain exhibits potential antioxidant and antiproliferative actions because it contains phytochemicals, such as phenolic acids, ferulic and sinapic acid, flavonoids, lignans, tocols, phytosterols, and folate, and these compounds have been shown to lower the risk of many diseases (14, 15).

Based on the evidence described above, the present study examined the neural dysfunction associated with hypothyroidism in female albino rats, and the effect of *Hordeum vulgare* (water soluble barley) administration on ameliorating these neural changes.

Materials and Methods

Chemicals

Carbimazole in the form of commercial tablets (Neo-Mercazole) obtained from AFT Pharmaceuticals was used. Barley was kindly provided from Agriculture Research Centre; Giza, Egypt.

Experimental animals

Adult female Wistar albino rats weighing 180-200 g, 6-8-week-old, were used in this study. Animals were purchased from the animal house of the National Organization for Drug Control and Research (NODCAR), Egypt and housed at 23±2°C and 55±5% humidity with a 12 hr light/dark cycle rats were provided a standard diet and water *ad libitum*.

Experimental design

Eighty adult female rats were randomly divided into equally four-treatment groups. Except for euthyroid animals (EU) (groups 1&2), hypothyroid animals (H) (group 3&4) were orally administered 5.0 $mg.kg^{-1}$ bwt Neo-Mercazole (16) until the end of the study. Following 30 days of Neo-Mercazole administration, groups 2 and 4 orally administered 100 $mg.Kg^{-1}$ bwt barley (B) (17) water suspension for four weeks. The four groups named: EU; EU+B; H; H+B. Following the 30 days of Neo-Mercazole administration the hypothyroidism status was manifested by increase in serum TSH level and decrease in fT_4 level as previously estimated in our work Abd-Rabo et al. (18)

Preparation of *Hordeum vulgare* (barley)

The used barley was identified and authenticated by Prof. Dr. Usama K. Abdel-Hameed; professor of plant taxonomy, Biology Department, Faculty of Science, Ain Shams University by aid of (19) *Hordeum vulgare* L. (Poaceae) -- Sp. Pl. 1: 84. 1753 (IK). Voucher specimens were deposited in the publicly available Herbarium of the Biology Department, Faculty of Science, Ain-Shams University.

Arabian folk remedies used soaked ground barley to reduce the antinutrient content, also to increase vitamin, mineral, protein and antioxidant levels, that make barley a useful treatment of many visceral disorders and sore throat(20, 21). Barley was prepared as an emulsion in water (one g ground barley soaked in 10 ml of distilled water) and administered daily *per* (17) . The nutritional facts of barley per 100 g is presented in Table (1).

Handling of the tissues

At the end of the experiment, the animals were anaes-

Table 1. nutrition facts in barley / 100 g.

Carbohydrates	(78.2 g)	Vitamin B6	(0.29 µg)	Choline	(38 mg)
Fibers	(15.5 g)	Vitamin K	(2.5 µg)	Riboflavin (B2)	(0.124 µg)
Energy	(350 Kcal)	Niacin(B3)	(4.8 µg)	Calcium	(30 mg)
Fat	(1.2 g)	Pantothenic acid (B5)	(0.29 µg)	Iron	(3.5 mg)
Protein	(10 g)	Thiamine (B1)	(0.2 µg)	Magnesium	(80 mg)
Vitamin A	(15 µg)	Folic acid	(25 µg)	Phosphorus	(200 mg)
				Zinc	(2.5 mg)
				Potassium	(250 mg)

thetized with 1% isoflurane followed by rapid decapitation (22).The whole brains were removed from 10 rats from each group and the hypothalamus, hippocampus, cerebral cortex, midbrain and cerebellum were dissected using a sharp blade. The other 10 rats were rapidly decapitated, whole brain and thyroid gland were immediately removed and stored in ice-cold saline at -20 °C for further biochemical and comet assay.

Determination of thyroid hormone levels in whole brain tissue homogenates

Level of fT4 was estimated using ELISA Kit using antibody specific for rat, supplied from WKEA Medical Supply Co. The TSH, TBG and TTR levels were determined using ELISA Kits specific for rats, supplied from Glory Science using antibodies specific for rats (Glory Science CATALOG #s: 30606, 30511 and 30701, respectively).

Determination of monoamines and free amino acids in different brain regions

Preparation of brain samples

The first step in the determination of monoamines and free amino acids involved weighing of different brain area (hypothalamus, hippocampus, cerebral cortex, midbrain and cerebrum) and homogenizing each region in ice 1/10 weight/volume 75% aqueous HPLC-grade methanol solution. The homogenate was centrifuged at 4000 g for 10 min, a portion of the clear supernatant samples of each different was further dried for free amino acid determination as described later (23).

Derivatization procedure for free amino acid determination

The derivatization procedure started by re-drying the test sample using a drying solution that consisted of a 2:2:1 mixture (by volume) of methanol: water: triethylamine (TEA). The drying solution was added to the dry sample, shaken well and then placed under vacuum until the sample was completely dry. The derivatizing agent consisted of a 7:1:1:1 mixture (by volume) of methanol: TEA: water: PITC. The derivatizing solution was added to the re-dried sample, shaken well, incubated at room temperature for 20 min, and then vacuum-dried (70 mbar). The dry sample was then diluted with a sample diluent composed of 0.71 g of disodium-hydrogen phosphate adjusted to a pH 7.4 using 10% phosphoric acid. Acetonitrile was then added to a 5% concentration by volume with the resulting solution. The derivatized samples and amino acid standards were injected (the injected volume was 20 µl) into the column for separation by HPLC.

HPLC condition for determination of monoamines and free amino acids

An Agilent HPLC system Model 1260, with a Rheodine injector, a 50-µl loop and a UV variable wavelength detector were used for monoamine assays. A clear supernatant sample were directly injected into an AQUA column with dimensions of 150×4.6 mm 5 µ C18, purchased from Phenomenex, USA and separated under the following conditions: a mobile phase of 97% 20 mM potassium phosphate, pH 3.0, and 3% methanol, a flow rate of 1.5 ml/min, and a UV wavelength of 270 nm. NE, DA, and 5-HT were separated after 10 min. The resulting chromatogram identified the position and concentration of each monoamine compared to the standard. Finally, the content of each monoamine was calculated using a previously described method and reported as µg/g brain (24) . For free amino acids, PICO- TAG column (Waters) was used 3.9 × 30 cm. The assay conditions were as follows: temperature: 46 °C; wavelength: 254 nm; and flow rate: 1 ml/min. The resulting chromatogram identified the position and concentration of each amino acid compared to the amino acid standard. Finally, the content of each amino acid was reported in µg/g brain region.

Comet assay in whole brain and thyroid tissues

A comet assay of whole brain and thyroid homogenates was performed using a previously reported method (25), with modifications. One gram of homogenized whole brain and thyroid tissues from each animal in their respective group was transferred to 1 ml of phosphate-buffered saline (PBS), stirred for 5 min and filtered. The cell suspension (100 µl) was mixed with 600 µl of low melting point agarose (0.8% in PBS), and then 100 µl of this mixture were spread on slides that had been pre-coated with normal melting point agarose. The coated slides were immersed in lyses buffer (0.045 M Tris/borate/EDTA buffer (TBE), pH 8.4, containing 2.5% sodium dodecyl sulphate (SDS)) for 15 min. Slides were placed in an electrophoresis chamber containing the same TBE buffer lacking SDS. The electrophoresis conditions were 2 V/cm for 2 min and 100 mA. Staining was achieved with 20 µg/ml ethidium bromide (EtBr) at 4 °C. The staining of humidified samples was observed. The DNA fragment migration patterns of 100 cells for each group level were evaluated under a fluorescence microscope (with excitation filters of 420-490 nm). The lengths of the comet tails were measured from the middle of the nucleus to the end of the tail at 40x magnification, and the size of the comet was measured. For visualization of DNA damage. The comets were captured with an Olympus fluorescence microscope equipped with a CCD camera, and the images were quantitatively evaluated for the percentage of DNA tail, tail length (µm), and tail moment using CASP software (Comet Assay Software Project 1.2.2).

Quantitative real-time polymerase chain reaction (qRT-PCR) for the detection of 5-HT and DA receptor expression in whole brain tissue homogenates

Gene expression was detected using qRT-PCR. Briefly, total RNA was extracted from frozen brain tissue samples using the RNeasy Mini Kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's protocol, and the extracted RNA was quantified by spectrophotometry. Real-time RT-PCR was performed on a Step One Plus system (Applied Biosystems, USA) to quantitatively assess mRNA expression, gene-specific primer pairs were designed with Gene Runner Software (Hasting Software, Inc., Hasting, NY, USA) from RNA sequences from the gene bank as presented in table (2), Data from real-time assays were calculated using the v1•7 sequence detection software from PE Biosystems (Foster City, CA, USA). Relative expression of studied

Table 2. Primer RNA sequences used for the analysis.

	Primer sequence
5-HT_{1A}	Forward primer 5'- GATCTCGCTCACTTGGCTCA -3' Reverse primer 5'- ACCTTCCTGACAGTCTTGCG -3'
5-HT_{2A}	Forward primer 5' CACCGACATGCCTCTCCATT -3' Reverse primer 5'- GGACACAGGCATGACAAGGA -3'
DA receptor	Forward primer 5'-GACAGTCCTGCCAAACCAGAGAA-3' Reverse primer 5'-TGGGCATGGTCTGGATCTCAAAGA-3'
Beta actin	Forward primer 5′ - CCTGTATGCCTCTGGTCGTA -3′ Reverse primer 5′ - CCATCTCTTGCTCGAAGTCT -3′

gene mRNA was calculated using the comparative Ct method. All values were normalized to the beta-actin which was used as the control housekeeping gene and reported as fold change over background levels detected in all groups (26).

Examination of Extracellular Signal Regulated Kinase (ERK1/2) level in whole brain tissues

ERK1/2 is a marker of phosphorylated cellular protein modulating the non-genomic function of fT4, it was determined using ELISA kit specific for rats (catalogue no. #:34246) according to the manufacturer's instruction (GSCIENCE Glory Science Co., Ltd. USA).

Examination of DNA-degradation product and apoptotic marker in the whole brain tissues

A DNA-degradation product 8-hydroxy-guanosine (8-OHdG) and apoptotic marker Casp-3 were determined using ELISA kit specific for rats (catalogue no. #:30088 and #:30186, respectively) according to manufacturer's instruction (GSCIENCE, Glory Science Co., Ltd. USA).

Statistical analysis

All biochemical data were statistically analysed using SPSS version 22. Data were expressed as mean ± S.D. Statistical differences between groups were performed using ANOVA test with significance level $p<0.05$, the *Post Hoc* was carried out using Tukey test.

Results

Effect of barley on thyroid hormone, transporter proteins and ERK1/2 levels whole brain tissue

In brain tissue Neo-mercazole significantly depleted the TH, TTR and ERK 1/2 levels in brain, while a slight significant increase in the TSH and TBG levels were observed compared with that in the EU and EU+B groups. Compared with the EU group, the EU+B group experienced a significant decrease in TTR and a significant increase in the TBG levels. The H+B group exhibited a significant increase in THs and ERK1/2 with a significant decrease in TSH levels, which reached the levels observed in the EU and EU+B groups. Regarding the transporter proteins, H+B significantly augmented the TTR level, but these levels remained lower than those observed in the EU and EU+B groups. The TBG levels were significantly increased in the H+B group, reaching the levels observed in the EU+B group and surpassing those observed in the EU group (Table 3). Collectively, these results indicate that barley maintains the TH and ERK 1/2 levels and balances the transporter proteins to normal controls levels.

Effect of barley on caspase-3 and 8-OHdG in whole brain tissues

Compared with the EU group, the hypothyroid (H) group exhibited a significant increase in cysteine-aspartic proteases-3 (caspase 3) and 8-hydroxy-2' -deoxyguanosine (8-OHdG) in brain tissue relative to EU group. The groups treated with barley exhibited a significant depletion in caspase-3 and 8-OHdG compared with that in the H group and reached normal control levels compared with EU group (Figure 1).

Effect of barley on DNA degradation in the thyroid and whole brain tissues

A comet assay was used to reveal the degree of DNA

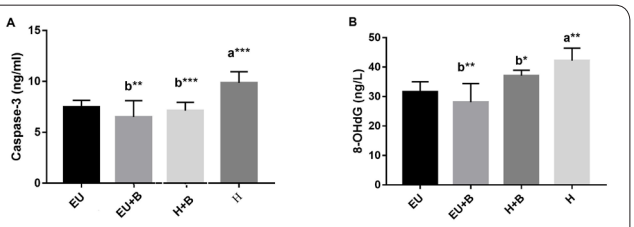

Figure 1. Effect of barley on **(A)** caspase-3 (ng/ml) and **(B)** 8-OHdG (ng/ml) in EU and H groups. All data represented by mean ± SD, n= 10 animals, *p<0.05, **p<0.01 and ***p<0.001,[a]: mean significance difference from control group. [b]: mean significance difference from hypothyroid group. [C]: mean significance difference from H+B.

Table 3. Effect of barley on THs, TTR and TBG in brain tissue of EU- and H-groups.

	EU	EU+B	H	H+B
fT4 (*μg/ml)*	4.43 ± 0.11	4.21 ±0.14	3.09 ±0.25 (a*)	4.46 ±0.19(b**)
TSH *(mIU/ml)*	12.47 ±0.42	12.73 ±0.62	15.97 ±0.26 (a*)	12.53 ±0.29 (b*)
TTR (ng/ml)	51.23 ±2.39	45.64 ±6.53 (a*)	16.85 ±1.88 (a***)	34.25 ±1.65 (a** b***)
TBG (pg/ml)	1.58 ± 0.04	2.35 ±0.16 (a**)	2.09 ±0.04 (a*)	2.57 ±0.14 (a***b*)
ERK 1/2 (pg/ml)	43.67 ±1.53	46.33 ±1.53(a*)	33.19 ± 2.16 (a**)	47.94 ± 0.60(b**)

All data represented by mean ± SD, n= 10 animals, *p<0.05, **p<0.01 and ***p<0.001,[a]: mean significance difference from control group.[b]: mean significance difference from hypothyroid group

damage. As DNA was degraded, it converted from a supercoiled form to a comet-like shape with a measurable tail length. The increased in the tail length of the DNA-comet shape reflects the degree of DNA damage. Our study revealed that compared with the EU and EU+B groups, the hypothyroid status induced a significant increase in the tail length, in the thyroid and brain tissues. In the EU group treated with barley, the tail length was maintained at normal levels compared with that in the EU group. H group treated with barley exhibited a significantly reduced tail length, but the length was increased compared with that in the EU groups. These results reveal that hypothyroidism induced considerable DNA damage, resulting in a marked increase in apoptosis based on the caspase-3 and 8-OHdG results. The treatment with barley mitigated the level of DNA damage. This effect was related to the suppression of caspase-3 in the H+B group, suggesting that barley has an anti-apoptotic and anti-oxidant effects. (Figures 2 and 3).

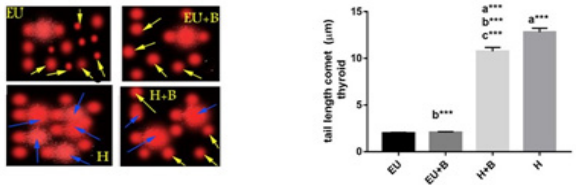

Figure 2. Effect of barley on DNA damage in thyroid tissue **(A)** fluorescence photomicrograph showing comets in EU-, H-, EU+B and H+B-groups. The → indicated the intact DNA and → indicated the degree of damaged DNA **(B)** Tail length expressed in µm in thyroid tissue of all treated groups. All data represented by mean ± SD, n= 10 animals, *p<0.05, **p<0.01 and ***p<0.001,[a]: mean significance difference from control group. [b]: mean significance difference from hypothyroid group.[C]: mean significance difference from H+B.

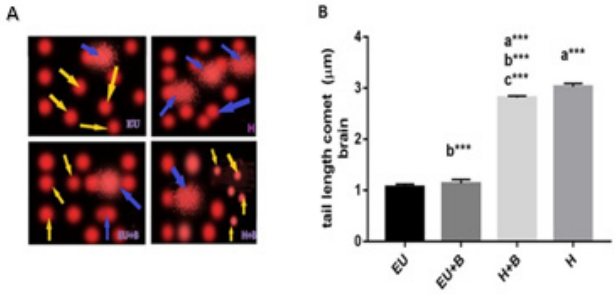

Figure 3. Effect of barley on DNA damage in whole brain tissue **(A)** fluorescence photomicrograph showing comets in EU-, H-, EU+B and H+B-groups. The → indicated the intact DNA and → indicated the degree of damaged DNA **(B)** Tail length expressed in µm in whole brain tissue of all treated groups. All data represented by mean ± SD, n= 10 animals, *p<0.05, **p<0.01 and ***p<0.001,[a]: mean significance difference from control group. [b]: mean significance difference from hypothyroid group.[C]: mean significance difference from H+B.

Effect of barley on neurotransmitter levels in different brain areas

The present study investigated the effect of hypothyroidism on the monoamine's levels in different brain areas, including the frontal cortex, hippocampus, hypothalamus, midbrain and cerebellum. As shown in Table (4), compared with the EU group, the DA, NE and 5-HT levels were significantly decreased in all brain areas in the H group. In the EU group treated with barley for four consecutive weeks, DA, NE and 5-HT levels were maintained at normal levels compared with those in the EU group. In addition, the H group treated with barley exhibited significantly augmented the levels of monoamines compared with those in the H group; however, these levels were reduced compared with those in the EU group.

Effect of barley on excitatory and inhibitory amino acids in whole brain tissues

Figure (4) reveals that the group treated with Neomercazole (H group) exhibited significantly increased levels pf inhibitory amino acids, including GABA, taurine, serine and glycine, compared with those in the EU group. Compared with the EU-group, the EU group treated with barley maintained the excitatory amino acid

Table 4. Effect of barley on neurotransmitters level in discrete brain regions in control and treated groups.

	Frontal Cortex	Hippocampus	Hypothalamus	Mid brain	Cerebellum
NE(µg g^{-1} tissue)					
EU	0.52 ± 0.03	0.69 ± 0.02	0.40 ± 0.01	0.70 ± 0.01	0.58 ± 0.01
EU+B	0.45 ± 0.02	0.65 ± 0.01	0.40 ± 0.01	0.69 ± 0.01	0.60 ± 0.01
H	0.21 ± 0.09^{a***}	0.31 ± 0.01^{a***}	0.13 ± 0.01^{a***}	0.35 ± 0.01^{a***}	0.29 ± 0.01^{a**}
H+B	0.35 ± 0.01^{a**b*}	0.46 ± 0.01^{a**b**}	0.22 ± 0.01^{a**b**}	0.48 ± 0.01^{a**b**}	0.40 ± 0.01^{a**b**}
DA(µg g^{-1} tissue)					
EU	0.59 ± 0.02	2.40 ± 0.07	1.473 ± 0.10	1.31 ± 0.01	0.60 ± 0.01
EU+B	0.55 ± 0.01	2.39 ± 0.09	1.32 ± 0.06	1.32 ± 0.01	0.60± 0.01
H	0.26 ± 0.01^{a***}	0.93 ± 0.03^{a***}	0.90± 0.03^{a*}	0.85 ± 0.01^{a**}	0.27 ± 0.05^{a***}
H+B	0.35 ± 0.01^{a**b**}	1.17 ± 0.01^{a**b**}	1.07 ± 0.02^{a*b*}	1.0 ± 0.01^{a*b*}	0.40 ± 0.01^{a**b**}
5-HT(µg g^{-1} tissue)					
EU	0.57 ± 0.01	0.38 ± 0.01	0.78 ± 0.01	0.72 ± 0.01	0.47 ± 0.01
EU+B	0.56 ± 0.02	0.38 ± 0.01	0.76 ± 0.02	0.65 ± 0.05	0.50 ± 0.01
H	0.23 ±0.01^{a***}	0.11 ± 0.01^{a***}	0.45 ± 0.01^{a**}	0.30 ± 0.01^{a***}	0.18 ± 0.01^{a***}
H+B	0.35 ± 0.01^{a**b**}	0.18±0.01^{a**b*}	0.54 ± 0.01^{a*b*}	0.41 ± 0.01^{a**b**}	0.28 ±0.01^{a**b**}

All data represented by mean ± SD, n= 10 animals, *p<0.05, **p<0.01 and ***p<0.001,[a]: mean significance difference from control group.[b]: mean significance difference from hypothyroid group.

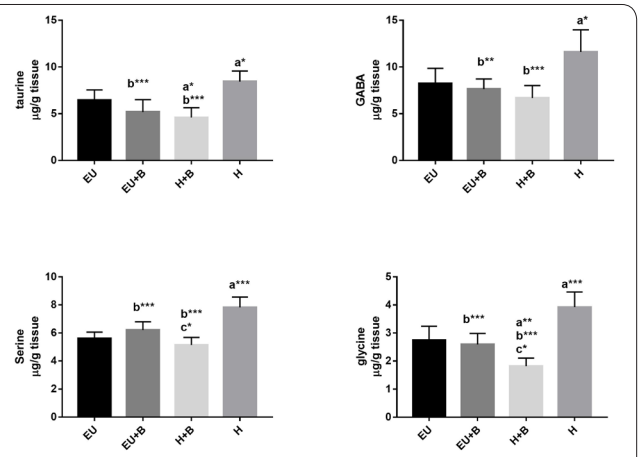

Figure 4. Effect of barley on inhibitory amino acids in EU and H groups, **A**) Taurine, **B**) GABA, **C**) serine and **D**) Glycine, All data represented by mean ± SD, n= 10 animals, *p<0.05, **p<0.01 and ***p<0.001,[a]: mean significance difference from control group. [b]: mean significance difference from hypothyroid group.[C]: mean significance difference from EU+B.

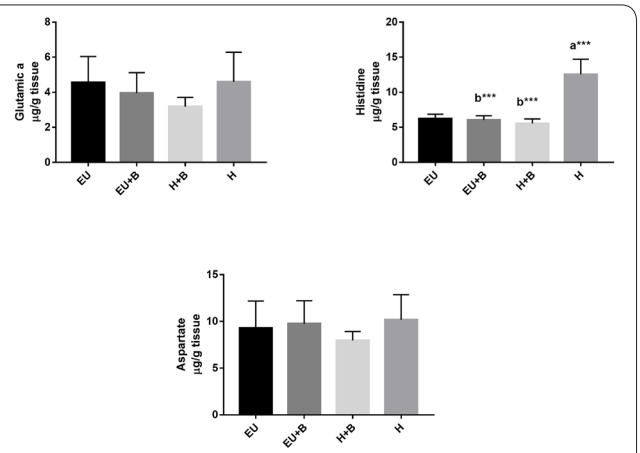

Figure 5. Effect of barley on excitatory amino acids in EU and H groups, **A**) Glutamic a, **B**) histidine and **C**) Aspartate. All data represented by mean ± SD, n= 10 animals, *p<0.05, **p<0.01 and ***p<0.001, [a]: mean significance difference from control group. [b]: mean significance difference from hypothyroid group. [C]: mean significance difference from EU+B.

levels. In addition, the barley treatment significantly attenuated the excitatory amino acid levels in the H-group compared with those in the untreated H group, achieving the levels in GABA and serine observed in the EU groups; however, taurine and glycine were slightly reduced compared with those in the EU group. Regarding the excitatory amino acids, the glutamate and aspartate levels were not record significantly altered in all assigned groups; however, the H group exhibited a significant increase in the histidine levels compared with that in the EU group. The barley treatment attenuated the increase in the histidine levels in the H group, attaining the normal levels observed in the EU and EU+B groups (Figure 5).

Effect of barley on 5-HT and DA receptor mRNA expression in whole brain tissue

The induction of hypothyroidism significantly augmented the dopamine receptor mRNA expression, and a significant decrease in 5-HT_{1A} and 5-HT_{2A} receptor mRNA expression, relative to that in the EU group was observed. The barley treatment resulted in the main-

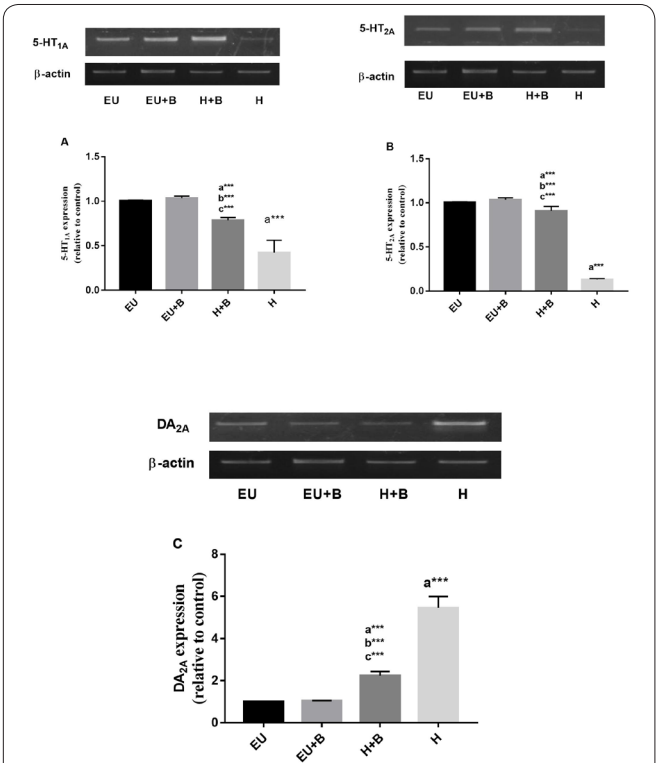

Figure 6. Effect of barley on relative expression of **A**) mRNA 5-HT_{1A}, **B**) mRNA 5-HT_{2A} and **C**) mRNA DA_{2A}. All data represented by mean ± SD, n= 10 animals, *p<0.05, **p<0.01 and ***p<0.001, [a]: mean significance difference from control group. [b]: mean significance difference from hypothyroid group. [C]: mean significance difference from EU+B.

tenance of the normal expression of 5-HT_{1A}, 5-HT_{2A} and DA_{2A} mRNA compared with the levels observed in the EU group, in which it significantly ameliorated the mRNA expression of the receptors in the H group. However, the levels did not reach those noted in the EU group. (Figure 6)

Discussion

In the present study, a hypothyroid model was created in adult female albino rats through the oral administration of Neo-mercazole for 30 days, which lead to elevated serum TSH levels and decreased fT_3 and fT_4 levels, as determined in our previous work (18).

Neo-mercazole is an antithyroid agent that blocks thyroid hormonogenesis by inhibiting the action of thyroid peroxidase (TPO) and preventing the formation of thyroglobulin from tyrosine, which interferes with the synthesis of THs. The TH levels in the brain are controlled by very effective regulatory mechanisms involving thyroid secretion, transport to the brain, and the expression of deiodinases. THs enter the brain either directly through the blood-brain barrier or via astrocytes and interstitial fluid or indirectly via the blood-CSF-barrier (27). The membrane transporters of TH belong to several families, including the monocarboxylate transporter (MCT) and TTR families, which are the primary proteins that transports T_4 in the brain and are secreted by the choroid plexus (CP) (27). The present model of hypothyroidism revealed a highly significant decrease in the T_4 levels and elevated TSH levels in brain tissues that could be attributed to changes in the TTR and TBG levels. These effects could be attributed to the effect

of Neo-mercazole blocking haeme iron from binding H_2O_2, leading to the deactivation of TPO enzyme, which is a rate limiting enzyme in the synthesis of TH (28).

Treatment with the water barely solution (*Hordeum vulgare*) for one-month improved TH and transporter levels to some extent, likely due to the increased iron (Fe) content in *Hordeum vulgare* (barley). This improvement was potentially dependent on the increased concentration of TPO, which is composed of Fe, as reported in previous studies (13, 29, 30). The improvement in the TTR levels induced by the *Hordeum vulgare* (barley) treatment is due to its high zinc content, which increases the transthyretin concentration, as mentioned in a previous study (31). In addition, *Hordeum vulgare* (barley) contains high levels of carbohydrates and tryptophan: branched chain amino acids (Trp:BCAA) (32). Oxidative stress (ROS) is defined as an imbalance between the production of pro-oxidant substances and antioxidant defences. Hypothyroidism augments the oxidative insult, impairing the brain by increasing nitric oxide (NO) and NO synthase (NOS) levels in hippocampus, which affects the lipid composition of rat tissues and induces DNA damage (33, 34). These findings are consistent with the present data, which revealed highly significant increases in oxidative stress marker (8-OHdG) and an apoptotic marker (Caspase -3) levels and are confirmed by alkaline comet assays of thyroid and brain tissue homogenates. Comet assays are used to determine the level, shape, size and extent of DNA damage in individual cells (35, 36). Damaged DNA containing single and/or double strand breaks is separated from the intact DNA (head) and generates a "comet" tail. The elevated percentage of tailed DNA in thyroid and brain tissues of the present hypothyroid model was reduced by the *Hordeum vulgare* (barley) treatment due to its high levels of antioxidant, including vitamins A and E, which repair cellular and DNA damage. Additionally, folic acid, which is a component of barley, exerts a highly potent effect on repairing DNA damage in the brain and thyroid because it is essential for nucleotide and DNA biosynthesis and DNA repair and methylation (7, 12, 13, 32). Additionally, the barley administration improved the oxidative stress levels given the high levels of zinc, vitamin E and main sources of antioxidants, such as phenolic acids, flavonoids and phytic acid, which exhibit free radical scavenging activity (20). Ferulic acid, which is the dominant phenolic compound in barley shows high antioxidant activity by absorbing and neutralizing oxygen radicals (14, 20), while zinc protects against oxidative stress by stabilizing membranes through the inhibition of the enzyme nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) and the stimulating of the synthesis of metallothioneins, which reduce the levels of hydroxyl radicals and sequester ROS produced in response to hypothyroidism.

Mitogen-activated protein kinases (MAPKs), including ERK1/2, regulate numerous cellular processes during brain development, including gene expression, migration, metabolism, differentiation, proliferation and apoptosis, by conveying signals from cell surface receptors to the nucleus, which triggers a genomic response in neurons and the integration of signals from other transduction pathways.THs play important roles in activating G protein-coupled receptors, which lead to ERK1/2 phosphorylation and cAMP response element (CRE)-mediated transcription of some proteins important for memory given that nongenomic actions of THs involve T_4 but not T_3 (11). Therefore, the depletion of ERK1/2 could be implicated in neural dysfunction following hypothyroidism (37). Consistently, the induction of hypothyroidism in the present study significantly and negatively affected ERK1/2 levels, which are correlated with reduced fT4 levels in brain tissue. The *Hordeum vulgare* (barley) administration improved ERK1/2 levels by increasing fT_4 levels in brain tissues, mediating its activation. Similar to humans, TH deficiency can impair neurogenesis, differentiation, maturation and synaptic transmission in the hippocampus of adult rats (38). THs interact with a broad range of neurotransmitters and are involved in regulating mood through post-receptor signalling and gene regulatory mechanisms. The present study estimated the levels of neurotransmitters in different brain areas to confirm this hypothesis. Based on the results, NE, DA and 5-HT concentrations were significantly reduced in all brain areas studied (cerebellum, midbrain, cerebral cortex, hypothalamus and hippocampus) in rats with hypothyroidism induced by Neo-mercazole. As previously described, this finding could be attributed to the reduced oestradiol level as described previously (18), which play a potent role in enhancing neurogenesis functions (39–42). The administration of *Hordeum vulgare* (barley) improved the disturbances in the dopaminergic, serotonergic and noradrenergic pathways in the brain induced by hypothyroidism via two different mechanisms. First, the high phytoestrol content modulate oestrogen receptors (ERα& β) expression and elevate oestradiol levels, which subsequently results in improvement in neurotransmitters, such as serotonin and dopamine, as mentioned in previous work (18). Second, barley is enriched with tryptophan and phenylalanine and could regulate the synthesis of 5-HT, DA and NE through the conversion of tryptophan to 5-hydroxytryptophan (5-HTP) to 5-HT and hydrolysis of phenylalanine to generate tyrosine that ultimately produces DA and NE (13, 32, 43, 44).

Consistent with the current results, Neo-mercazole evoked a significant reduction in the serum DA, 5-HT and NE levels in adult hypothyroid rats. Folic acid treatment dose not exert any beneficial effect on either DA or 5-HT levels but significantly increased NE levels (45). Moreover, several studies have reported that reduced TH levels are associated with dopaminergic and serotonergic dysfunction (7, 9, 46). In addition, the inhibition of the deiodinase enzyme in the brain of patients with depression leads to reduced T_3 levels and subsequently reduced 5-HT secretion (47). Any fluctuation in the plasma levels of certain amino acids leads to the changes in the levels of neurotransmitter precursors (48). GABA, which is the main inhibitory neurotransmitter, does not cross the blood-brain barrier. Several studies have demonstrated the an increase in GABA levels is negatively correlated with blood oxygen-glucose levels in various brain region, which may lead to ischemia and induce cell death potentially involving apoptosis (49–51). In the present study, hypothyroidism induced a significant increase inhibitory amino acid, including GABA and histidine, which is excitatory amino acid. These results could explain the increase in caspase-3, which may be

attributed to reduced blood oxygen-glucose levels in several brain regions as a result of increased GABA levels. Furthermore, Wiens and Trudeau (52) documented that hypothyroidism increases GABA levels in the adult brain, the authors attributed this finding to the effect of THs on the transport mechanisms as well as enzyme activities for GABA synthesis and degradation, all of which are sensitive to thyroidal state.

Patients with depressive symptoms displayed increased glycine and taurine levels compared with those of controls, reflecting the impaired uptake, synthesis and transport of these amino acids across the blood-brain barrier (53). The *Hordeum vulgare* (barley) treatment in the present study caused a renormalization the observed disturbances in the amino acid and neurotransmitter levels. Barley is enriched with folic acid, which is involved in the synthesis of monoamine neurotransmitters and serotonergic, dopaminergic and noradrenergic systems by acting as a cofactor for enzymes that convert tryptophan to 5-HT and enzymes that convert tyrosine to noradrenaline(7).

During serotonergic neurotransmission, serotonin is released into the synaptic cleft and exerts its action on the postsynaptic neuron. Then, a portion of serotonin is transported again into the presynaptic neuron by specific transporters to reincorporate the vesicles, and another portion is metabolized by the monoamine oxidase (MAO) to form 5HIAA. In the rat's brain, 5-TH neuron cell bodies are concentrated in raphe nuclei of midbrain, and their axons reach the entire brain at a very high density, similar to that in the hippocampus. Dopamine and serotonin receptors are a widely group of G protein-coupled receptors in the rat brain, suggesting that dopamine and 5-TH have many central effects (54). The present study investigated the effect of hypothyroidism on dopamine and serotonin subtype receptors and recorded an elevation in concentration of dopamine receptor, whereas serotonin receptors decreased significantly. An increase of dopamine receptors was noted, whereas serotonin receptors were significantly decreased. The alteration in serotonin receptor densities was restored by the barley administration, given its enriched levels of tryptophan, which is metabolized to serotonin (32, 44) and activates these receptors. The elevation in serotonin levels after barley administration in the present study also resulted in ERK1/2 improvement in brain tissue, which was reduced by hypothyroidism induction. The binding of serotonin to 5-TH2 receptors stimulates ERK1/2 phosphorylation via the release of epidermal growth factor (EGF) agonist and transactivation of (EFG) receptors (55).

The gonadal hormone oestradiol modulates mesolimbic dopamine systems, which are crucial for the expression of motivated behaviours in the female rats, given that binding to its membrane receptors (mER) rapidly modulates dopamine (56). Abd-Rabo et al. (18) estimated the level of serum oestradiol in hypothyroid- and barley-treated groups. The results revealed elevated oestradiol levels in the groups treated with barley, which could explain the positive effect of barley had on restoring dopamine levels in the present study. Crider and Pillai (57) clarified the potential therapeutic effect of oestrogen and ERβ in some neurodevelopmental disorders, such as anxiety, locomotion, fear, memory and learning. These neuroprotective effects of oestrogen occur through genomic and non-genomic signalling and antioxidant functions.

The reproductive hormones oestrogen and progesterone modulate the dysregulated serotonergic, dopaminergic, and glutamatergic neurotransmission by regulating the expression of receptors and the synthesis, reuptake, and release of the neurotransmitter serotonin and dopamine, which interact with dopaminergic neurons directly to downregulate D2 autoreceptors and indirectly by inhibiting GABAergic transmission (58). This finding is consistence with those of the present study, given that *Hordeum vulgare* with its high tryptophan and phenylalanine levels was exhibited an increase in a gonadal hormones, as reported in a previous work (18).

Based on the above findings, barley (*Hordeum vulgare*) is a nutritious food with high carbohydrate, zinc, magnesium content and a high amino acids Trp:BCAA ratio. These important constituents explain the positive effect of barley on ameliorating the neural dysfunction induced by hypothyroidism. Additionally, barley (*Hordeum vulgare*) is recommended for relieving stress and improving mood and depression.

Authors' contributions

All authors contribute to design the protocol. Dr. L.F. Wahman and M.M. Abd-Rabo were responsible for gathering and acquisition the data and writing and editing the manuscript. Dr.M.M. Abd-Rabo carried out the statistical analysis of data. Dr. Magda HM Youseef & lobna F. wahman and Marwa M. Abd- Rabo interpreted the data. Magda HM Youseef was responsible for revising the manuscript

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

All data are available without restrictions with Dr/ Marwa M. Abd- Rabo ; Assistant prof of Biochemistry; Biology and Hormonal Evaluation Department marwa10mokhtar1@outlook.com

Consent for publication

Not Applicable.

Ethical approval

All the experimental procedure of the study was approved by the Institutional Animal Care and Utilization Committee (IACUC) at the National Herpetology & Tropical Medicine Research Institute, Egypt (reg. no. IRB00003916).

Funding

There has been no funding for this work.

Acknowledgments

The authors would like to thank and appreciate Prof. Dr. Laila I. Abou-Basha for her scientifically assistance.

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