Histopathological and immunohistochemical alterations in the hippocampus of thyroidectomized rats and the role of hemin and ketoconazole in treatment

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Abstract—Background: The thyroid hormones are recognized as key metabolic hormones that play a critical role in brain development, mediate important effects within the CNS throughout life and regulate the metabolism and functions of various neurotransmitters. The hippocampus is a major component of the rat brains, where it belongs to the limbic system and plays important roles in long term memory and spatial navigation. In Alzheimer’s disease, the hippocampus is one of the first regions of the brain to suffer damage; memory problems and disorientation appear among the first symptoms. Hemeoxygenase (HO) is a heme-catabolizing enzyme that converts heme into biliverdin, iron and carbon monoxide. Promising therapeutic effects of increased brain HO-1 levels have been reported in models of neurodegenerative disorders and brain infection including Alzheimer disease (AD), Parkinson disease (PD), and malaria. The present work aimed to declare the effect of low thyroid hormone status on hippocampus tissue histopathological and immunohistochemical changes of p53 and caspase. Also, the present study aimed to elucidate the role of hemin and ketoconazole (KTZ) supplementation as a HO inducer and inhibitor respectively. Material and method: Seventy male Wistar rats were divided into the following groups: GI, Control group; GII, Thyroidectomized group; GIII, Sham operated group; GIV, Thyroidectomized & Hemin group; GV, Thyroidectomized & KTZ group; GVI, Sham operated & Hemin group and GVII, Sham operated & KTZ group. Results: the results obtained revealed various histopathological changes showed a large number of damages neurons, diffuse vacuolar degeneration, degenerating (i.e., shrunken, argyrophilic) neurons with reduction and distortion of the pyramidal cells in the hippocampus of thyroidectomized rats when compared with the hippocampus of control or sham rats. Hippocampus coronal sections in thyroidectomized rat that treated with hemin revealed little tissue injury with mild neuronal damage and a few diffuse vacuolar degeneration. There were a moderate to strong positive reactions for p53-ir (grade 3) and casp-ir (grade 4) were detected in hippocampus on thyroidectomized rat group. A marked decreased (grade 1) and (grade 2) in the intensity of p53-ir and caspase 3 respectively on the hippocampus in thyroidectomized rat that treated with hemin when compared with thyroidectomized rat. Conclusion: our results revealed that hemin has an ameliorating role in hippocampus of thyroidectomized rats by improving the histopathological alteration and the intensity of p53 and caspase 3.

Index Terms — Hemeoxygenase, Thyroidectomized, hippocampus, Hemin, Ketoconazole, P53, Caspase 3.

1 INTRODUCTION

Normal brain development requires the presence of thyroid hormones that are essential for cell migration, dendrite and axon outgrowth, synapse formation, myelination and gliogenesis [1]. A thyroidectomy is an operation that involves the surgical removal of all or part of the thyroid gland. Hypothyroidism is an underactive thyroid gland that cannot make enough thyroid hormone to keep the body running normally [2]. Neurologically, hypothyroidism has been associated with cerebellar ataxia, confusion, delusions, memory impairment, hallucinations and psychotic behavior [3]. Thyroid hormones are known to modulate a number of neurotransmitter systems [4].

The hippocampus is a major component of the rat brains, where it belongs to the limbic system and plays important roles in long term memory and spatial navigation [5]. Damage to the hippocampus can also result from oxygen starvation (hypoxia), encephalitis, or medial temporal lobe epilepsy. People with extensive hippocampal damage may experience amnesia, i.e. the inability to form or retain new memories [6].

Hemeoxygenase is a membrane-bound enzyme responsible for catalyzing the first and rate-limiting step in the degradation of heme [7]. HO-1 expression and activity are highly induced by numerous factors such as heavy metals and ultraviolet radiation, also by infrared, hypoxia, cytokines, hemin, nitric oxide and angiotensin II [8, 9]. The different functions of HO-1 are mediated by protective effects of the HO reaction products carbon monoxide (CO) and biliverdin/bilirubin like: antioxidant effect, antiprotective effect, anti-inflammatory effect, antiproliferative effect, vasoactive effect, anticoagulative effect and proangiogenic effect [10].

Hemin is a potent inducer of HO_1 in the different tissues. Recently, the antifungal agent KTZ has been reported to have antitumoreffects in prostate cancer [11]. The azole antifungal drugs share structural features with a series of novel hemeoxygenase (HO) inhibitors. The azole-containing antifungal drugs are potent HO inhibitors and that was showed in Kinobe et al. [12].

2 Materials and method
The experiments were performed on 70 male Wistar rats weighing 120 ± 10 g and of 6-7 week’s age. They were obtained from Serum and Vaccine Farm-Helwan-Egypt. The rats were kept in the laboratory for one week before the experimental work and maintained on a standard rodent diet (20% casein, 15% corn oil, 55% corn starch, 5% salt mixture and 5% vitaminized starch; Egyptian Company of Oils and Soap Kafr-Elzayat Egypt) and water available ad libitum. The temperature in the animal room was maintained at 23 ± 2°C with a relative humidity of 55 ± 5%. Light was on a 12:12 light-dark cycle. The experimental protocol was approved by Local Ethics Committee and Animals Research. The rats were randomly and equally divided into seven groups (ten animals each):

- Group I (Control group)
  - Rats were fed on standardized diet ad libitum.
- Group II (thyroidectomized group)
  - Rats were subjected to surgery of thyroidectomy.
- Group III (Sham operated group)
  - Rats were subjected to sham operation.
- Group IV (Thyroidectomized & Hemin group)
  - Rats were subjected to surgery of thyroidectomy like group II and after four weeks rats were orally treated with hemin by a stomach tube (15 mg/kg/day) for four weeks [13].
- Group V (Thyroidectomized & Ketoconazole (KTZ) group)
  - Rats were subjected to surgery of thyroidectomy and after four weeks rats were orally treated with KTZ by a stomach tube (100 µmol/kg/day) for four weeks [12].
- Group VI (Sham operated & Hemin group)
  - Rats were subjected to sham operation like group III and after four weeks rats were orally treated with hemin.
- Group VII (Sham operated & Ketoconazole (KTZ) group)
  - Rats were subjected to sham operation and after four weeks rats were orally treated with KTZ.

Thyroidectomy was performed on rats anesthetized with intraperitoneal injection with sodium pentobarbital and subjected to a complete necropsy according to Tenorio-Velasquez et al., [14] method. Briefly, by using a stereomicroscope (Zeiss, Germany) for better observation, the stenothyroid muscle was carefully dissected out to avoid injury to the laryngeal nerve and was complete excised. After surgery, ketorolac (Sintex-Mexico) (50 mg/kg) and gentamicin (Shering Plough-Mexico) (10 mg/kg) were administered over 5 days to alleviate pain and prevent infection. At the end of the experimental period, rats were euthanized with intraperitoneal injection with sodium pentobarbital and subjected to a complete necropsy.

### 2.1 Histological investigation

The skulls were opened with fine scissors and the brains of different groups under study were separated immediately after dissection then hippocampus is sliced and washed in saline. Hippocampus was fixed in 10% neutral formalin for histopathological studies. The pieces of hippocampus were processed and embedded in paraffin wax according to standard procedures. Paraffin sections (5µm thick) were mounted on gelatin chromalum–coated glass slides and used for Hae-matoxylin and eosin stains as a routine method for investigation.

Tissues were put in a rinsing solution then immediately removed taking care to handle specimens gently to minimize trauma and put into fixative solution (10% neutral formalin) for 24 hrs and then washed in running tap water for 15 minutes. Tissues were dehydrated in standard alcholic series (70% alcohol for 6-24 h, 90% alcohol overnight, 95% alcohol (2 changes 15 minutes each, then absolute alcohol 3h). Tissues were cleaned in xyline (2 changes 10 min. each). Then were embedding in paraffin wax (3 changes 30 min. each at 60°C), then fresh paraffin wax for one hour. Tissues were sectioned 4-6 µm thickness and stained with Harris Hematoxyline for 5 min. Differantiated in 1% acidic ethanol for 30 seconds then Blueing agent was added. Tissues were stained with eosin for 2-5 minutes then washed by water until the desired shades of red or pink were obtained and then dehydrated in 90%, 96% and 100% ethanol for 5 minutes. Tissue was cleaned in two changes of xylene 5 minutes each or longer for better cleaning. Sections were covered with clean cover slips then dried and microscopically examined [15].

### 2.2 Immunohistochemical detection of p53 in hippocampus tissue

Expression of p53 proteins was detected using avidin Biotin Complex (ABC) method [16, 17] for hippocampus in different group under study. Dewaxed and rehydrated sections were washed in distilled water for 5 min, rinsed in PBST for 10 min and incubated with 10% normal goat serum for 15 min to reduce non-specific background staining. Then, the sections were incubated with anti-rabbit p53 monoclonal antibody (Dako, 1:80) for 1-2 hours at room temperature. The sections after 5 baths in PBST were incubated with biotinylated goat anti-rabbitimmunoglobulin (Nichirei, Tokyo, Japan). The sections after 5 baths in PBST were further incubated with Avidin Biotin Complex (ABC: Nichirei, Tokyo, Japan) for 1 hour at RT. The reaction was developed by using 20 mg 3,3′-diaminobenzidine tetrahydrochloride (DAB, Wako pure chemical industries, Ltd) in 40 ml PBST, pH 7.2 containing 10 ml of hydrogen peroxide (H2O2) for 7-9 min at a dark room followed by distilled water then dehydrated and mounted. The criterion for a positive reaction confirming the presence of p53 proteins is a dark, brownish, intracytoplasmic precipitate. For the negative control, the primary antibody was omitted to guard against any false positive results which might develop.

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from a non-specific reaction. Negative control sections were done by substituting p53 primary antibodies by normal serum of goat.

2.3 Immunohistochemical detection of caspase3 in hippocampus tissue

Brain sections were incubated in blocking solution (PBS containing 5% normal donkey serum, 2% Triton X-100, 0.02% bovine serum albumin, BSA) overnight at 4°C and left overnight at 4°C in staining solution (PBS containing 5% normal goat serum, 0.25% Triton X-100, 0.02% BSA) with primary antibodies, including mouse monoclonal antibody to GFAP (Invitrogen); rabbit polyclonal antibody to doublecortin (Abcam); goat monoclonal antibody to ionized calcium-binding adaptor molecule-1 (Iba-1, Abcam); and rabbit polyclonal antibody to active caspase 3 (R&D Systems).

Sections were then incubated in staining solution containing Hoechst33258 (Invitrogen, 2 μg/ml), Fluorescein isothiocyanate-conjugated donkey anti-mouse IgG and RRX-conjugated donkey anti-rabbit IgG or cy5-conjugated donkey anti-goat IgG (1:200; Jackson Immunoresearch) in the dark overnight at 4°C. Sections were then washed in PBS and mounted with Aqua Poly/Mount (Polyscience Inc., Warrington, PA, USA) at 4°C. Sections were then examined by mean of a research microscope. Brightness, contrast were adjusted using Adobe Photoshop software (version 4.0.1; Adobe Systems, Mountain View, CA). Image analysis was adjusted using PAX-it image analysis software.

3 Results

3.1 Histopathological findings in the hippocampus

No histological changes were observed in the CA1, CA2 and CA3 regions of hippocampus of control group. Coronal sections in hippocampus of control, sham, sham & hemin and sham & KTZ groups revealed a normal hippocampus structure (Figs. 1-4 respectively). The histopathological examination of thyroidectomized rat brains revealed various histopathological changes. This histopathological changes showed a large number of damages neurons, diffuse vacuolar degeneration, degenerating (i.e., shrunken, argyrophilic) neurons with reduction and distortion of the pyramidal cells were observed in the hippocampus when compared with the hippocampus of control or sham rats (Figs. 5&6). Hippocampus coronal sections in thyroidectomized rat that treated with hemin revealed little tissue injury with mild neuronal damage and a few diffuse vacuolar degeneration (Fig. 7) while hippocampus sections in thyroidectomized rat that treated with KTZ revealed moderate tissue injury with diffuse vacuolar degeneration and moderate neurofibrillary degeneration damages neurons (Fig. 8).

3.2 Immunohistochemical results:

3.2.1 P53 immunohistochemical results:

The detection and distribution of P53 immunoreactivity (P53-ir) in the hippocampus in coronal sections in control, sham, sham & hemin and sham & KTZ groups revealed negative re-
et al. [24] have published a series of studies characterizing the effect of developmental hypothyroidism on a variety of anatomical features, including spine density of pyramidal neurons in the cerebral cortex, the organization of callosal connections, and other features.

Also, during critical periods of development, hypothyroidism causes abnormalities of the CNS such as incomplete maturation of neuronal and glial cells, reduction in synaptic densities and myelin deficits [25]. Thyroid hormone deficiency during a brief perinatal period produces severe neurological defects in humans and experimental animals [26]. In our results hippocampus coronal sections in thyroidectomized rat that treated with hemin revealed little tissue injury with mild neuronal damage and a few diffuse vacuolar degeneration and this finding is agreed with Fouad et al. [27] who reported that hemin significantly improved renal histopathological examination of the APAP-induced tubular damage. Thyroid hormones are closely associated with neuronal apoptosis [28, 29]. Caspases are a family of cysteine proteases and act as the executors of regulating cell apoptosis. As one of the key initiators, caspase-3 is involved in the mitochondria-mediated apoptotic pathway [30]. Caspase-3 is the major effector caspase involved in apoptotic pathways and is thought to trigger the execution phase of apoptosis [31]. Caspase-3 activation is considered to be a specific marker of apoptosis induction. Hypothyroidism during development enhanced apoptosis in the hippocampus [29]. As well, the number of cleaved caspase-3-positive cells in the hippocampal dentate gyrus was increased by hypothyroidism induced by maternal methimazole (MMI), indicating that hypothyroidism accelerated apoptotic neuronal cell death in the hippocampal dentate gyrus [32] and this finding is accepted with our study where there was a strong positive reactions for Cas-ir (grade 4) were detected in hippocampus of thyroidectomized group. There was a marked decrease (grade 2) in the intensity of Cas-ir on the hippocampus in thyroidectomized rat that treated with hemin when compared with thyroidectomized rat and this is in agreement with Guan et al. [33] who stated that the increase in caspase-3 levels was blocked by hemin pre treatment in hippocampus injury after acute carbon monoxide poisoning. Similarly, hemin pre-treatment inhibited neuronal death. Guan et al. [33] suggest that up regulation of HO-1 in the hippocampus of CO-exposed rats can inhibit the induction of caspase-3 activity and protein expression, where asSnPP (inhibitor of HO activity) administration abrogated this effect. It was indicated that the anti-apoptotic properties resulting from HO-1 induction contributed much to the neuroprotective effect.

There are numerous external signals that are involved in the regulation of the apoptosis. P53 is the one of most extensively investigated pathways [34]. Apoptosis may be initiated in any phase of the cell cycle, but the majority of cells undergo apoptosis in the G1 phase [35]. P53 acts on the G1/S checkpoint. In our results there was a moderate to strong positive reactions for p53-ir (grade 3) were detected in hippocampus on thyroidectomized rat brains group and this finding is agreed with Alva-Sánchez et al. [23] who stated that histological analysis showed that hypothyroid animals exhibit significant neuronal damage in all regions of the hippocampus accompanied by the triggering of the apoptotic pathway (increases in p53, p21 and the Bax/Bcl-2 index) and no changes in proliferation (Cyclin D1 and PCNA). A marked decrease (grade 1) in the intensity of p53-ir on the hippocampus in thyroidectomized rat that treated with hemin when compared with thyroidectomized rat.

ROS generation is a key factor in the activation of the p53 signaling pathway by many chemotherapeutic drugs or oxidative conditions, intracellular molecular targets of upstream signaling that regulate p53 expression are not fully defined. Furthermore, increased HO-1 activity results in the degradation of the heme moiety, a potentially toxic pro-oxidant, and the production of bilirubin, an antioxidant capable of scavenging peroxyl radicals and inhibiting lipid peroxidation. Therefore, we hypothesized that HO itself or its catalytic by-products such as biliverdin, CO, and free iron might mediate the expression or activation of p53. Our data is not in agreement with Liu et al. [36] who have revealed that direct exposure of CO has an apoptotic effect by inhibiting both expression of p53 and release of mitochondrial cytochrome c in vascular smooth muscle cells. However, he also demonstrated that overexpression of HO-1 induces a marked increase in p53 expression in the same cells [37]. As well, Lee et al., [38] reported that HO activity is associated with regulatory mechanisms for p53 expression in the human retinal pigment epithelium (RPE) cell line ARPE-19.

Cobalt protoporphyrin (CoPP), an activator of HO, increased the expression of p53 in RPE cells while zinc protoporphyrin ZnPP, an inhibitor of HO, Although ZnPP increased the level of HO-1 protein while inhibiting HO activity, ZnPP inhibited the expression of basal p53 in ARPE-19 cells [38].
Figs. (9-16): Photomicrographs of hippocampus stained with P53-ir in groups under study. Figs. 9-12: Revealed a negative reaction for P53-ir (grade 0) in pyramidal cells in control group, sham operated group, sham operated group treated with hemin and sham operated group treated with KTZ respectively. Figs. 13&14: Revealed a moderate to strong positive reactions for p53-ir (grade 3) in thyroidec-tomized group. Fig. 15: Revealed a marked decreased reaction for p53-ir (grade 1) in thyroidec-tomized treated hemin group. Fig. 16: Revealed a mild decreased reaction for p53-ir (grade 2) on hippocampus coronal sections in thyroidec-tomized treated KTZ group.

Figs. (17-24): Photomicrographs of hippocampus stained with Cas-ir in groups under study. Figs. 17-20 respectively revealed a negative reaction for Cas-ir (grade 0) in pyramidal cells in control group, sham operated group, sham operated group treated with hemin and sham operated group treated with KTZ respectively. Figs. 21&22 revealed a strong positive reaction for Cas-ir (grade 4) in thyroidec-tomized group. Figs. 23 revealed a marked decreased reaction (grade 2) in the intensity of Cas-ir reaction in thyroidec-tomized treated with hemin group. Fig. 24 revealed a mild decrease in the intensity of Cas-ir reaction (grade 3) on hippocampus coronal sections in thyroidec-tomized treated KTZ group.

REFERENCES


