# 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 p35 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 cas-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 ameroliating role in hippocampus of thyroidectomized rats by improving the histopathological alternation and the intensity of p35 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 enzymeresponsible 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, antiapoptotic 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, theantifungal 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 on70 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 thelaboratory for one week before the experimental work andmaintained on a standard rodent diet (20% casein, 15% corn oil,55% corn starch, 5% salt mixture and 5% vitaminzed starch; Egyptian Company of Oils and Soap Kafr-Elzayat Egypt) and water available adlibitum. The temperature in the animal room was maintained at23 ± 2 8C with a relative humidity of 55 ± 5%. Light was on a 12:12 hlight -dark cycle. The experimental protocol was approved by Local Ethics Committee and Animals Research. The rats were randomly andequally 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 cut and the trachea was exposed. The parathyroid gland was located, dissected from the thyroid gland, and reimplanted into the surrounding neck muscle. The thyroid gland was carefully dissected out to avoid injury to the laryngeal nerve and was complete lyexcised. After surgery, ketorolac (Sintex-Mexico) (50 mg/kg) and gentamicin (Shering Plough-Mexico) (10 mg/kg) were administered over 5 day 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.


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### 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 Haematoxylin 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 alcoholic 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 xylene (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. Differentiated 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 antirabbitimmuoglobulin (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

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) [18].The prepared sections were 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 P53immunoreactivity (P53-ir) in the hippocampus in coronal sections in control, sham, sham &hemin and sham & KTZ groups revealed negative reaction for P53-ir (grade 0) in pyramidal cells (Figs. 9-12 respectively). Moderate to strong positive reactions for p53-ir (grade 3) were detected in hippocampus on thyroidectomized rat group (Figs. 13&14). A marked decrease (grade 1) in the intensity of p53-ir on the hippocampus in thyroidectomized rat that treated with hemin (Fig. 15) when compared with thyroidectomized rat. The intensity of p53 -ir was mild decreased (grade 2) on hippocampus coronal sections in thyroidectomized rat that treated with KTZ (Fig. 16) when compared with hippocampus coronal sections in thyroidectomized rat.

#### 3.2.2 Caspase 3 immunohistochemical results:

The detection and distribution of caspase immune reactivity (Cas-ir) in the hippocampus in coronal sections in control, sham, sham &hemin and sham & KTZ groups revealed negative reaction for Cas-ir (grade 0) in pyramidal cells (Figs. 17-20 respectively). Strong positive reactions for Cas-ir (grade 4) were detected in hippocampus on thyroidectomized rat brains group (Figs. 21&22). A marked decrease (grade 2) in the intensity of Cas-ir on the hippocampus in thyroidectomized rat that treated with hemin (Fig. 23) when compared with thyroidectomized rat. The intensity of Cas-ir was mild decreased (grade 3) on hippocampus coronal sections in thyroidectomized rat that treated with KTZ (Fig. 24) when compared with hippocampus coronal sections in thyroidectomized rat.

## 4 Discussion

Hypothyroidism is caused by deficient thyroid hormone secretion. The hippocampus exhibits morphological plasticity well into adulthood. It is one of the few brain regions that retain the ability to make new neurons throughout adult life in several mammalian species including humans [19]. Transcription of the HO-1 gene is exquisitely sensitive to induction by prooxidant stressors and its up regulation is widely accepted as a biomarker of oxidative stress [20]. 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 [21]. Many studies have characterized the neuroanatomical consequencesof developmental hypothyroidism. In our results there was a various histopathological changes in the hippocampus of thyroidectomized group showed a large number of damages neurons, diffuse vacuolar degeneration, degenerating (i.e., shrunken, argyrophilic) neurons with reduction and distortion of the pyramidal cells when compared with the hippocampus of control or sham rats and this is compatible with Tousson et al. [22] who demonstrated moderate inflammation, edema, diffuse vacuolar degeneration, reduction and distortion in the pyramidal cells were observed in the hippocampus of hypothyroid rats.

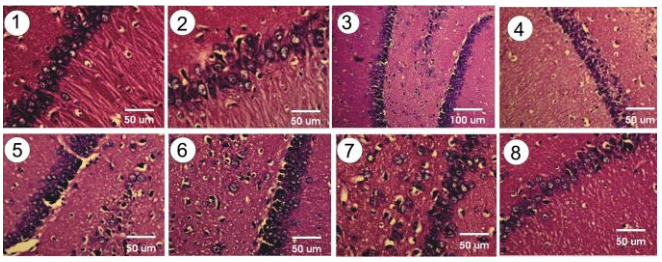
Also, studies by Ahmed et al. [2, 4] reported that thyroid hormone deficiency results in multiple morphological alterations in brain of the neonatal rats. Also, histological analysis showed that hypothyroid animals exhibit significant neuronal damage in all regions of the hippocampus [23]. As well, Berbel

et al. [24] have published a series of studiescharacterizing the effect of developmental hypothyroidism ona variety of anatomical features, including spine density of pyramidalneurones in the cerebral cortex, the organization of callosalconnections, and other features.

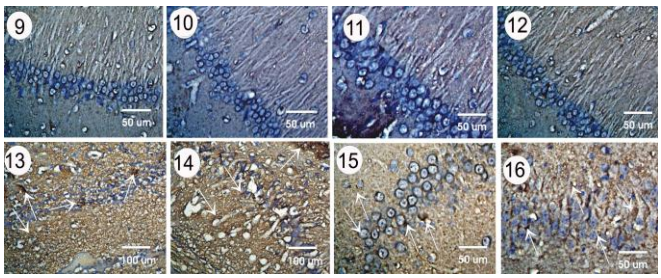
Also, during critical periods of development, hypothyroidism causesabnormalities of the CNS such as incomplete maturation of neuronal and glial cells, reduction in synaptic densitiesandmyelin deficits [25]. Thyroid hormone deficiencyduring a brief perinatal period produces severe neurological defectsin 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 decreased (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 heminpre treatment in hippocampus injury after acute carbon monoxide poisoning. Similarly, hemin pretreatment 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.

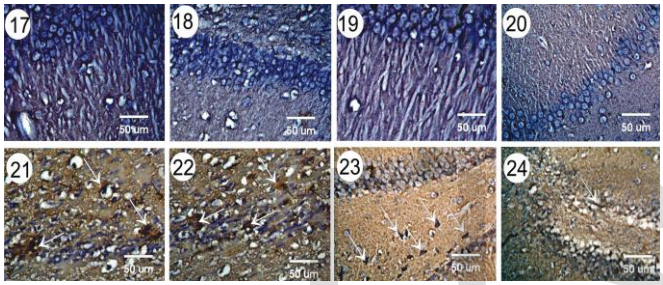
ROS generation is a key factor in the activation of the p53 signaling pathway by many chemotherapeutic drugs or oxidative conditions, intracellular molecular targets of upstream signaling that regulate p53 expression are not fully defined. Furthermore, increased HO-1 activity results in the degradation of the heme moiety, a potentially toxic pro-oxidant, and the production of bilirubin, an antioxidant capable of scavenging peroxy 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 aggrement 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 protoporphyrinZnPP, 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. (1-8): Photomicrographs of the hippocampus stained by HE in groups under study. Figs. 1-4: showed normal structure in control group, sham operated group, sham operated group treated with hemin and sham operated group treated with KTZ respectively. Figs. 5&6: showed a large number of damages neurons, diffuse vacuolar degeneration, neurons with reduction and distortion of the pyramidal cells in thyriodectomized group. Fig. 7: Revealed little tissue injury with mild neuronal damage and a few diffuse vacuolar degeneration in thyroidectomized treated hemin group. Figs. 8: Revealed moderate tissue injury with diffuse vacuolar degeneration and moderate neurofibrillary degeneration damages neurons in thyroidectomized treated KTZ group.




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 thyroidectomized group. Fig. 15: Revealed a marked decreased reaction for p53-ir (grade 1) in thyroidectomized treated hemin group. (Fig. 16: Revealed a mild decreased reaction for p53-ir (grade 2) on hippocampus coronal sections in thyroidectomized 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 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 thyroidectomized group. Figs. 23 revealed a marked decrease (grade 2) in the intensity of Cas-ir reaction in thyroidectomized treated with hemin group. (Fig. 24 revealed a mild decrease in the intensity of Cas-ir reaction (grade 3) on hippocampus coronal sections in thyroidectomized treated with KTZ group.

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