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Research Article

Ameliorating Effect of Ashwagandha (Withania Somnifera) Extract on Hippocampus and Growth Plate Changes Associated With Propylthiouracil Induced Hypothyroidism in Juvenile Rats

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ABSTRACT

Background: Hypothyroidism is a decrease in the production of the thyroid hormones and leads to gland dysfunction. Ashwagandha extract was used as an ayurvedic treatment and supposed to be as antihypothyroidism agent.
Objectives: to investigate the impact of ashwagandha (Ash) extract on propylthiouracil (PTU)-induced hypothyroidism in rats.
Subjects and Methods: The rats were divided into three groups, control group, PTU (hypothyroid) group (6mg/kg/day by oral route), PTU (6mg/kg/day by oral route) +Ash (50mg/kg/day by oral route) treated group. All treatment continued for 30 days. At the end of experiment, measurement of serum T3, T4 and TSH was performed. Thyroid gland, right sided tibia and dentate gyrus region of hippocampus were examined using histological, histochemical and immunohistochemical studies. All measurements were statistically analyzed.
Results: Decrease in serum T3 and T4 and congestion of the blood capillaries, follicular distortion, and vacuolar degeneration of some follicular cells were exhibited in thyroid gland of hypothyroid group. Histological changes in growth plate cartilage in the form of decrease of matrix deposition and plate thickness were detected. Dentate gyrus showed distorted granule cell layer. Immunohistochemically, low expression of the GFAP was expressed in astrocytes. PTU+Ash treated group showed improvement of the previous changes.
Conclusion: Administration of ashwagandha with PTU displayed protective effect on the thyroid gland and its associated histological changes in growth plate cartilage and dentate gyrus. Higher doses of ashwagandha should be used for extrapolation if it may give better results than the used dose.

Introduction

An important endocrine gland is the thyroid gland. It is extremely important to the body's metabolism and energy expenditure (1). Triiodothyronine (T3) and thyroxin (T4) hormones are made, stored, and released (2). The hormone T3 is the most active, while thyroxin is the most predominate. Deiodination of T4 results in the production of triiodothyronine (3, 4). The appropriate physiological and developmental processes can be maintained and controlled by thyroid hormones (5). For thyroid hormones to be properly formed there must be a sufficient quantity of iodine (6). The Hypothalamus-Pituitary-Thyroid Axis is responsible for controlling the biosynthesis and production of thyroid hormones. Therefore, any problem with this axis leads to aberrant thyroid hormone levels. This is described as either a hormonal over- or under-secretion that negatively affects (7, 8).

The clinical condition of hypothyroidism is one of the most frequent thyroid illnesses. Thyroid-stimulating hormone (TSH) levels are high in hypothyroidism, and the follicular cells are overstimulated, which causes alterations in metabolism since T3 and T4 synthesis is inadequate (9-11). One of the medications that cause hypothyroidism is propylthiouracil (12-14). A thiocarbamide-derived medication called propylthiouracil (six-n-propyl-2-thiouracil, or PTU) is used as an anti-thyroid agent (15). PTU is regarded as one of the key substances that might cause hypothyroidism by lowering T3 and T4 levels in the blood and raising TSH levels by inhibiting peripheral deiodinase and hypo-peroxidase (16).

The ayurveda system of traditional medicine uses withania somnifera, also known as ashwagandha, for a variety of medicinal purposes. Ashwagandha has been shown to be a safe and palatable herb by numerous toxicological studies (17). Aphrodisiac, adaptogen, liver tonic, antioxidant, antibacterial, and anti-inflammatory properties are only a few of the pharmacological benefits of ashwagandha that have been documented (18). Ashwagandha's well-documented pharmacological benefits also include the restoration of physiological and metabolic parameters, enhancement of cognitive function in geriatric situations, anti-arthritic, anti-aging, and recovery from neurodegenerative diseases (19). Withaferin A and sitoindosides VII-X are two of withania somnifera's active ingredients. These substances have antioxidative properties, as shown by the activation of endogenous catalase and superoxide dismutase, the increase in vitamin C, and the decrease in lipid peroxidation (20).

In ayurvedic literature, ashwagandha roots were referred to as Medhya Rasayana, or "helpful to the brain," and were employed as a tonic for the nervous system and in preventive health (21). Amyloid beta (A) and scopolamine-induced memory loss are both prevented by the root extract of W. somnifera, which improves cognitive function and increases the capacity of mental retention associated with diabetes (22, 23). Recent studies have shown that supplementing withanolide-enriched extract of W. somnifera root prevented memory impairment brought on by hypoxia, as well as the depletion of brain glutathione and free radical-scavenging enzymes, by altering the level of corticosteroid in the brain via the nitric oxide-cyclooxygenase-prostaglandin pathways (24).

According to a theory put forth (25), ashwagandha may enhance cholinergic activity, which may explain its therapeutic potential for amnesia and other neuroplasticity problems that are continuously impacted by stress and ageing. It is also known that W. somnifera root extract increases hippocampus cholinergic activity (23).

The goal of the current investigation was to determine whether ashwagandha might treat adolescent albino rats' hypothyroidism caused by PTU and any resulting alterations to the hippocampus and growth plate.

Subjects and Methods

Chemicals

- Ashwagandha extract (Ash) capsules were purchased from Now Food Company, USA. Each tablet contains 450 mg of the active ingredient.
- Propylthiouracil (PTU) is available under the name of Thyrocil® (Amoun Pharmaceutical Co., Egypt) as white discoid tablets containing 50 mg of the active ingredient.
- Both drugs were dissolved in normal saline and given orally by intragastric tube.

Laboratory animals

From the animal house at Faculty of medicine, Fayoum university, thirty juvenile (six weeks old) male Wistar albino rats (Rattus norvegicus), weighing 90-120, were taken. The animals were raised in tidy, well-ventilated cages with fresh wood shavings as bedding. Maintaining a 25°C temperature and a 12-hour light/dark cycle was done. The animals had unrestricted access to water and pellets of typical rodent diet. We made every attempt to limit animal suffering and employ only the amount of animals required to generate valid scientific data. Research Ethics Committee at faculty of medicine, Fayoum University accepted the experimental procedure, and all animal studies abide by the National Institutes of Health's guidance for the care and use of Laboratory Animals (NIH Publications No. 8023, amended 1985).

Determination of repeated acute drug toxicity of ashwagandha

Acute toxicity of ashwagandha was determined according to the Nuffield Council on Bioethics, (2005). Animals were given 50 mg/kg body weight of ashwagandha (Ash) by oral route for 30 consecutive days.

Experimental design

Three groups of ten rats each were formed from the rats, as follows: Rats in the control group were given saline solution. Rats in the PTU-group were given 6 mg/kg/day of (26) propylthiouracil orally. Rats in PTU+Ash group received oral doses of both propylthiouracil (6 mg/kg/day) (26) and ashwagandha extract (Ash) (50 mg/kg/day) (27). For a total of 30 days, the therapies were administered orally while using a gastric tube.

Sample collection

The rats were injected intraperitoneally by sodium thiopental for anesthesia at a dose of 50 mg/kg at the end of the experiment. Before scarification, retro-orbital puncture blood samples were collected and centrifuged. Before being tested for T3, T4, and TSH, the supernatant serum was separated into equal portions (about 200 µl) and frozen at -80. For the histological analysis, the thyroid glands, brain and right-sided tibia bones were separated.

Histological procedures

Thyroid gland and Brain samples were fixed in 10% formalin solution at room temperature for 24h. Samples were embedded in paraffin wax. Paraffin sections 5µm thick were stained with Hematoxylin and Eosin. Bone was decalcified using 10% EDTA for 5 days and processed into paraffin blocks, from which 7-µm-thick sections were cut and stained with H&E and PAS reaction. Brain tissue was also stained with cresyl violet and glial fibrillary acidic protein (GFAP) rat monoclonal antibody, (ThermoScientific, USA, ready to use,7 ml), (catalog no. 14-9892-95). Positive control was brain.

Hormonal assays: Serum triiodothronine (T3), thyroxin (T4) and thyroid-stimulating hormone (TSH) were measured by electrochemiluminescence immunoassay on a Cobas® e601 immunoassay analyzer (Roche-Hitachi Diagnostics, Mannheim, Germany). The hormones were assessed according to manufacturer's instructions using commercially available kits from Biodiagnostics (Cairo, Egypt).

Morphometric measurements

The measurements were done using the image analyzer computer system (Leica Qwin 500C) (Leica, England). The following measurements were performed:

- The mean thickness of growth plate cartilage in H & E stained sections.
- The mean area % and the optical density of PAS reaction and cresyl violet stain were measured in 10 non-overlapping low power fields (x10).
- The mean area % of immunoreactive cells for GFAP was measured in 10 non-overlapping high power fields (x40).

Statistical analysis

Mean values and the standard error reported as numerical data. GraphPad Prism (version 5.0, GraphPad Software, San Diego, CA, USA) was used to conduct all statistical analysis. Data were analyzed statistically using one-way ANOVA after checking for normality of distribution followed by post hoc multiple comparisons (Tukey's test) for a comparative study between the groups. P<0·05 was regarded as statistically significant.

Results

Effect of ashwagandha on acute toxicity

Administration of 50 mg/kg of ashwagandha to rats did not induce any toxicity, as evident by 100% survival of treated animals besides biochemical and microscopic results of thyroid, bone and hippocampus tissues as compared with the normal control.

Biochemical results

Results of the thyroid hormones level (T3, T4, and TSH) of the control group are represented in (Table 1). Treatment of rats with ashwagandha extract revealed significant improvement in hormone levels as compared with control and PTU group. In induced hypothyroidism, serum level of T3 and T4 was significantly decreased, while the level of TSH increased significantly as compared to the control group.

Table 1: Serum levels of T3, T4, TSH

	Control group	PUT group	Ashwagandha+PUT group
T3 (ng/ml)	5.8±0.2	2.9±0.6*	4.2±1.1*#
T4 (nmol/ml)	44.6±2.1	22.5±3.4*	35.9±1.34*#
TSH (µl/ml)	0.64±0.02	1.23±0.16*	0.89±0.04*#

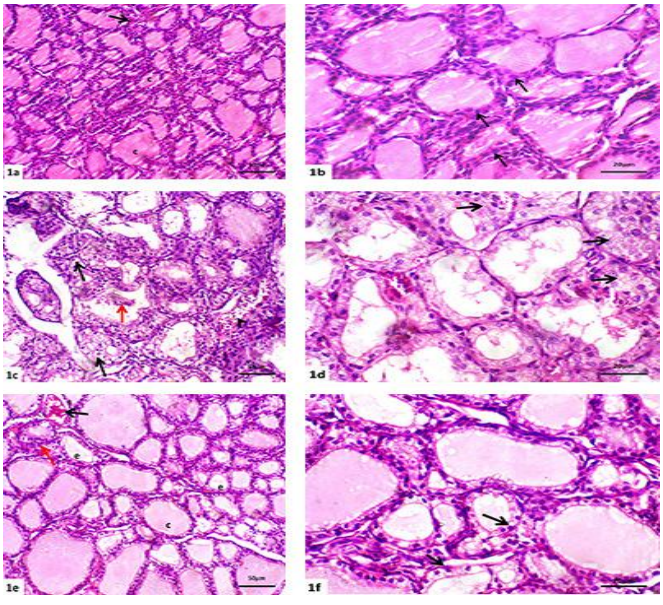
Data were expressed as mean values ± SE with significant difference when P value ≤0.05.
*significant difference as compared with control
significant difference as compared with PUT

Histological results

Thyroid gland histological results

Hematoxylin and eosin stained sections of control group revealed thyroid follicle of variable sizes. Their cavities were filled with acidophilic colloid. Minute blood capillary was observed between thyroid follicles. The lining epithelium of thyroid follicles was in the form of flattened to cubical cells with oval rounded vesicular nuclei (Fig. 1a, b). Propylthiouracil group showed disrupted and fused thyroid follicles. Congested blood vessel and many vacuolated follicular cells with dark nuclei were seen. Desquamated follicular cells were seen inside the lumen of some follicles. Stratification of lining epithelium of many follicles with loss of colloid was noticed (Fig. 1c, d). Therapeutic group revealed restored architecture of thyroid follicles with variable sizes. Some follicles were filled with colloid while others were still empty. Congested blood vessel and stratification of lining epithelium of some follicles were observed. Most follicles were lined with flat to cubical epithelial cells. Some cells were still showing vacuolated cytoplasm and dark nuclei (Fig. 1e, f).

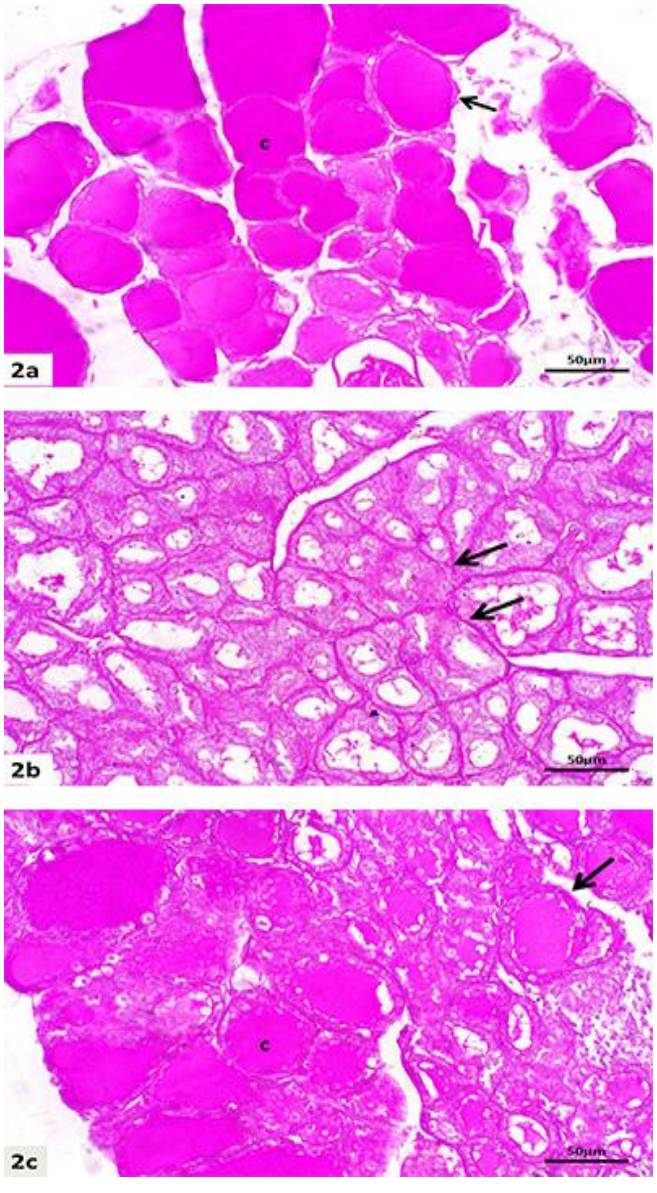
PAS stained sections of control group showed PAS positive reaction of colloid and basement membrane of thyroid follicles (Fig. 2a). While propylthiouracil group revealed many follicles with negative PAS reaction of colloid and positive reaction in the basement membrane of the follicles (Fig. 2b). Therapeutic group showed PAS positive reaction of the colloid and basement membrane of thyroid follicles and negative reaction in some follicles and at the peripheral vacuolation of colloid (Fig.2 c).



Figures (1a-f): Photomicrographs of H & E stained sections in thyroid gland from (a-b): control group showing (a): thyroid follicle of variable sizes. Their cavities are filled with acidophilic colloid (c). Minute blood capillary is noted between thyroid follicles (arrow). (b): lining epithelium of thyroid follicles in the form of flattened to cubical cells with oval rounded vesicular nuclei (arrows). (c-d): PTU group showing (c): disrupted and fused thyroid follicles. Congested blood vessel (arrow head) and many vacuolated follicular cells with dark nuclei (arrows) are seen. Desquamated follicular cells are seen inside the lumen of some follicles (red arrow). (d): stratification of lining epithelium of many follicles with loss of colloid (arrows). (e-f): PTU+Ash group showing (e): restored architecture of thyroid follicles with variable sizes. Some follicles are filled with colloid (c) while others are still empty (e). Congested blood vessel (arrow) and stratification of lining epithelium of some follicles is still noted (red arrow). (f): most follicles are lined with flat to cubical epithelial cells. Some cells still show vacuolated cytoplasm and dark nuclei (arrows). (1a, c, e x200- scale bar 50µm; 1b, d, f x400- scale bar 20 µm).

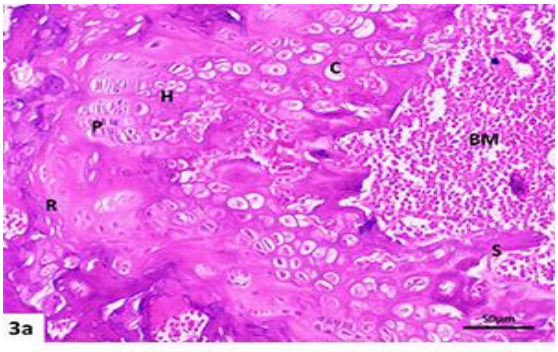
Epiphyseal tibia growth plate histological results

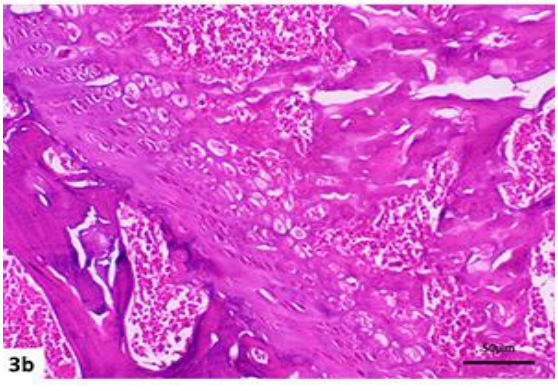
Hematoxylin and eosin stained sections of control group showed well organized epiphyseal cartilage forming the different zones: reserve zone (RZ), proliferating zone (PZ) (columns of proliferating chondrocytes), hypertrophic zone (HZ) (enlarged chondrocytes), and calcification zone (CZ) (with empty lacunae and surrounding basophilia of calcified matrix. Primary spongiosa were formed of trabecular specules and intervening bone marrow cavities (Fig. 3a). Propylthiouracil group revealed disorganized epiphyseal cartilage, disruption in chondrocyte columnar arrangement, and reduction in chondrocyte number in proliferative zone. Trabeculae of primary spongiosa were separated with multiple bone marrow cavities. Apparent reduced thickness of growth plate was observed (Fig. 3b). Therapeutic group showed well organized epiphyseal cartilage plate and preservation of resting zone and increased number of stacked chondrocytes. Some areas of cartilage plate were devoid of chondrocytes (Fig. 3c).

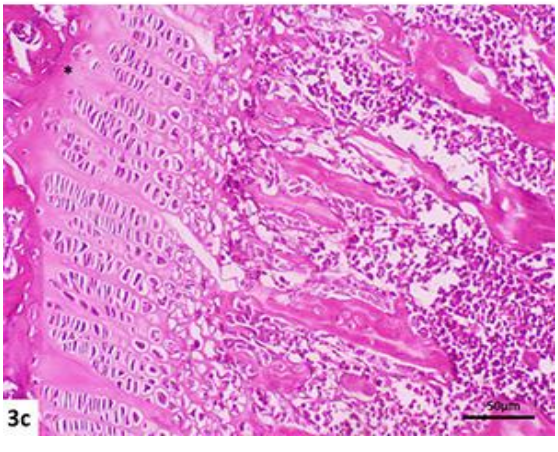


Figures. (2a-c): Photomicrographs of PAS stained sections in thyroid gland from (a): control group showing PAS positive reaction of colloid (c) and basement membrane of thyroid follicles (arrow). (b): PTU group showing many follicles with negative PAS reaction of colloid. Note the positive reaction in the basement membrane of the follicles (arrows). (c): PTU+Ash group showing PAS positive reaction of the colloid (c) and basement membrane (arrow) of thyroid follicles. Negative reaction is noted in some follicles and at the peripheral vacuolation of colloid. (2a-c x200- scale bar 50µm).

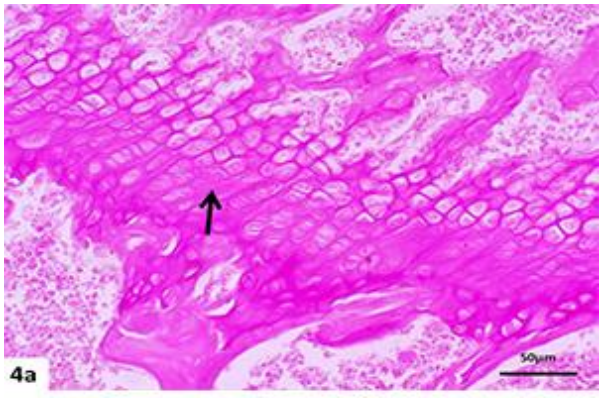
PAS stained sections of control group revealed strong positive PAS reaction in the matrix (Fig. 4a). While propylthiouracil group showed diminished areas of PAS positive reaction in the matrix (Fig. 4b). Therapeutic group showed strong PAS positive reaction in the matrix (Fig. 4c).

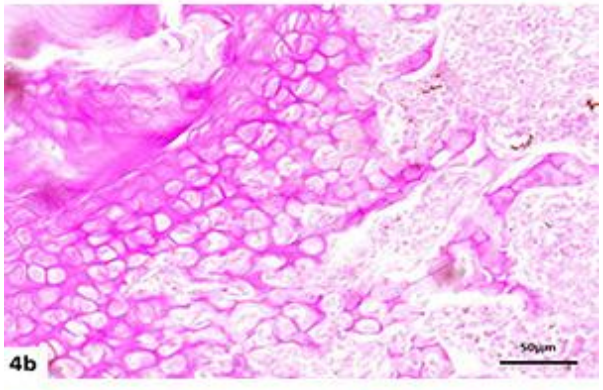


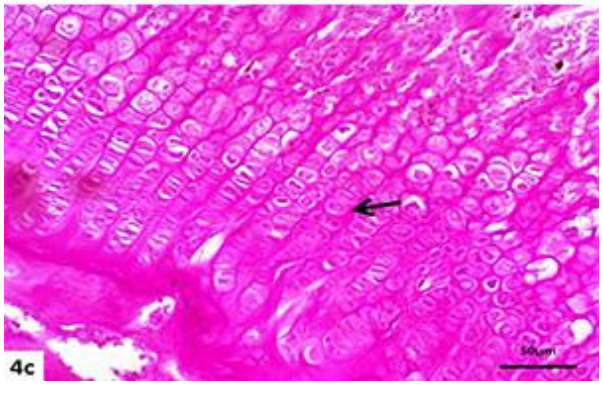




Figures. (3a-c): Photomicrographs of H & E stained sections in epiphyseal growth plate of tibia from (a): control group showing well organized epiphyseal cartilage forming the different zones: reserve zone (R), proliferative zone (P) (columns of proliferating chondrocytes). Hypertrophy zone (H) (enlarged chondrocytes), calcification zone (C) (with empty lacunae and surrounding basophilia of calcified matrix. Primary spongiosa (S) are formed of trabecular spicules and intervening bone marrow (BM) cavities. (b): PTU group showing disorganized epiphyseal cartilage, disruption in chondrocyte columnar arrangement, and reduction in chondrocyte number in proliferative zone. Trabeculae of primary spongiosa are separated with multiple bone marrow cavities. Note the reduced thickness of growth plate. (c): PTU+Ash group showing well organized epiphyseal cartilage plate and preservation of resting zone and increased number of stacked chondrocytes. Some areas of cartilage plate are devoid of chondrocytes (*). (3a-c x200- scale bar 50µm)





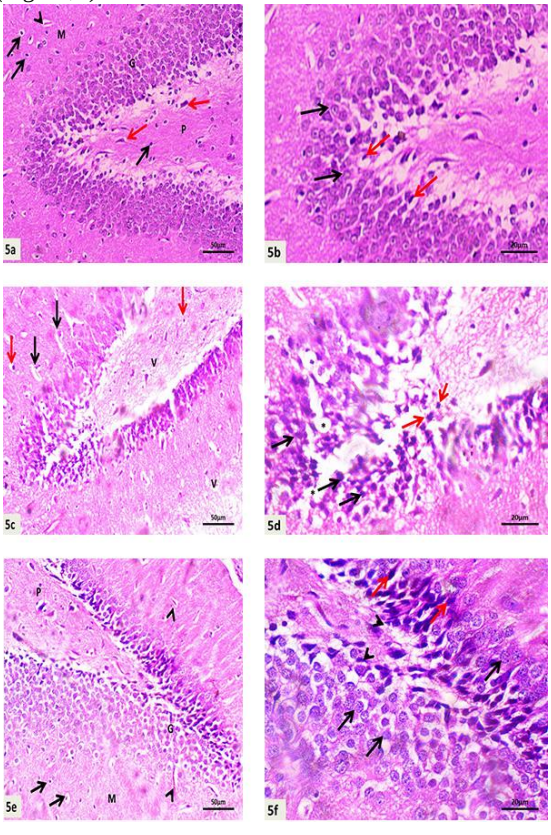


Figures (4a-c): Photomicrographs of PAS stained sections in epiphyseal growth plate of tibia from (a): control group showing positive PAS reaction in the matrix (arrow). (b): PTU group showing diminished areas of PAS positive reaction in the matrix. (c): PTU+Ash group showing strong PAS positive reaction in the matrix (arrow). (4a-c x200- scale bar 50µm)

Hippocampus (Dentate gyrus region) histological results

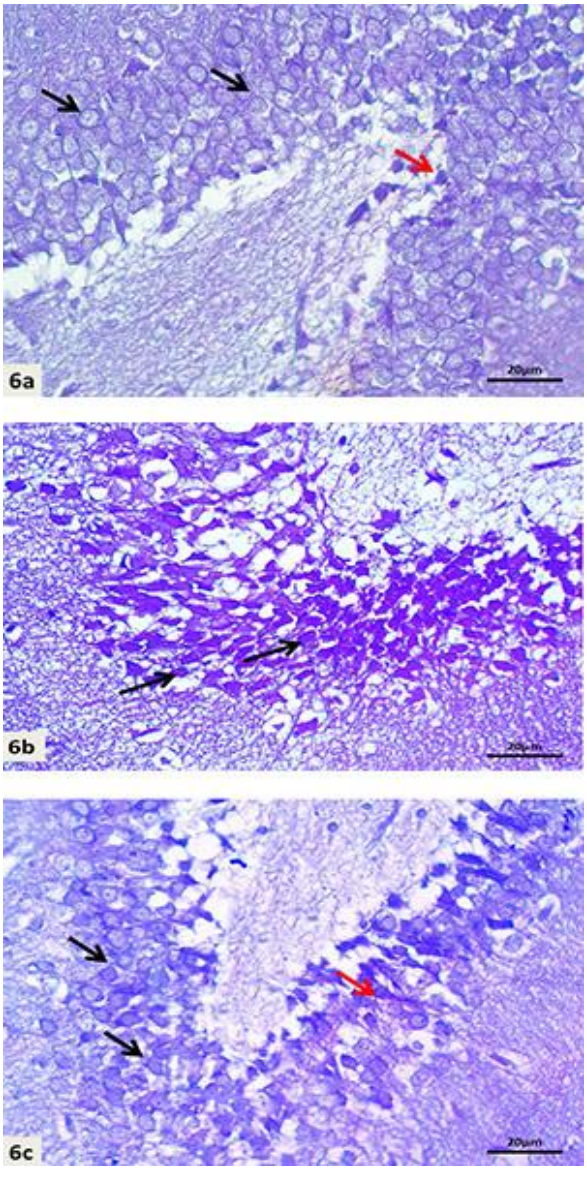
Hematoxylin and eosin stained sections of control group revealed three layers; molecular (ML), granule cell (GL) and polymorphic layers (PL). The glial cells (astrocytes), blood capillaries and pyramidal cells were observed. Granule cells appeared with rounded vesicular nuclei. Small spindle shaped cells were seen in the sub-granular zone (Fig. 5a, b). Propylthiouracil

group showed disturbed granule cell layer, dilated capillaries, many astrocytes with small dark nuclei and vacuolated neuropil (v). Degenerated granule cells with small dark nuclei and areas of cell loss in the granular layer were revealed. Many spindle shaped cells in sub-granular zone are noticed (Fig. 5c, d). Therapeutic group showed the three layers; molecular (ML), granule cell (GL) and polymorphic layers (PL). Astrocytes, blood capillaries and pyramidal cells were seen. Granule cells appeared with rounded vesicular nuclei, others had small dark nuclei in the granule cell layer. Many spindle shaped dark cells were noticed in sub-granular zone (Fig. 5e, f).



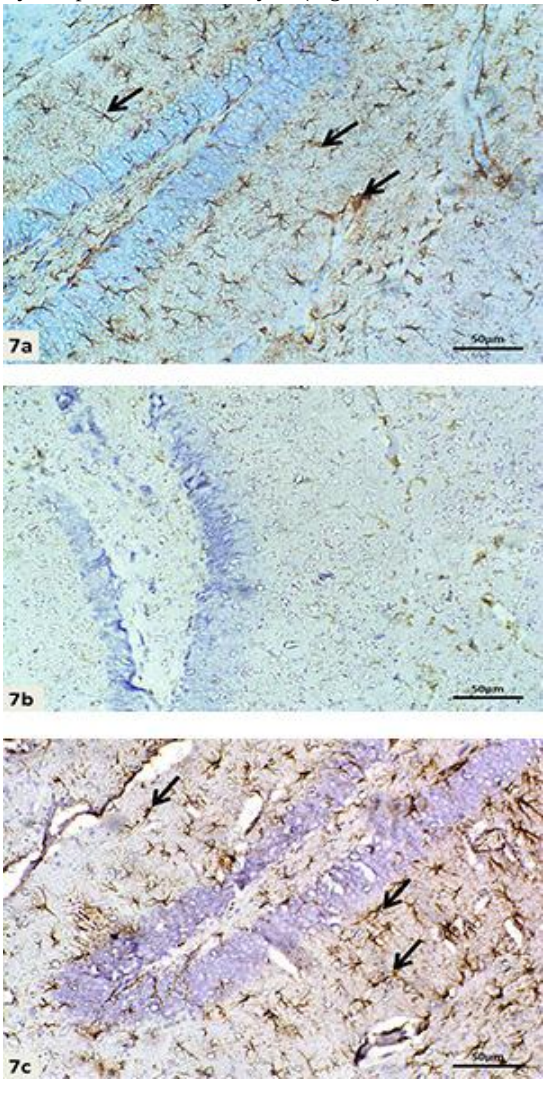
Figures. (5a-f): Photomicrographs of H & E stained sections of rat hippocampus (dentate gyrus) of (a-b): control group showing (a): three layers; molecular (M), granule cell (G) and polymorphic layers (P). Note the astrocytes (arrows), blood capillaries (arrow head) and pyramidal cells (red arrows) (b): Granule cells have rounded vesicular nuclei (arrows). Small spindle shaped cells (red arrows) are seen in the sub-granular zone. (c-d): PTU group showing (c): disturbed granule cell layer, dilated capillaries, many astrocytes with small dark nuclei and vacuolated neuropil (v). (d): Degenerated granule cells with small dark nuclei (arrows) and areas of cell loss (*) in the granular layer. Many spindle shaped cells (red arrows) in sub-granular zone are noticed. (e-f): PTU+Ash group showing (e): the three layer; molecular (M), granule cell (G) and polymorphic layers (P). Note the astrocytes (arrows), blood capillaries (arrow heads) as compared to control group. (f): Granule cells with rounded vesicular nuclei (arrows), others have small dark nuclei in the granule cell layer (red arrows). Many spindle shaped dark cells are noticed in sub-granular zone (arrow heads). (5a, c, e x200- scale bar 50µm; 5b, d,f x400- scale bar 20 µm)

Cresyl violet stained sections of control group showed purple Nissl's granules in perikarya of granule cells around their vesicular nuclei (Fig. 6a). Propylthiouracil group revealed ill-defined Nissl's granules in perikarya of granule cells with very dense nuclei (Fig. 6b). Therapeutic group revealed purple Nissl's granules in perikarya of granule cells around their vesicular nuclei. Some cells show ill-defined Nissl's granules (Fig. 6c).



Figures. (6a-c): Photomicrographs of cresyl violet stained sections of rat hippocampus (dentate gyrus) of (a): control group showing purple Nissl's granules (arrows) in perikarya of granule cells around their nuclei. Note the ill-defined Nissl's granules in the spindle immature neurons in the sub-granular zone (red arrow). (b): PTU group showing ill-defined Nissl's granules (arrows) in perikarya of granule cells (c): PTU+Ash group showing purple Nissl's granules (arrows) in perikarya of granule cells around their nuclei. Some cells show ill-defined Nissl's granules (red arrow). (6a-c x 400- scale bar 20µm)

GFAP immunostained sections of control group showed strong positive cytoplasmic immune reaction in the cell body and processes of astrocytes (Fig. 7a). While, propylthiouracil group revealed weak immunoreactivity in the astrocytes (Fig. 7b). Therapeutic group showed strong positive cytoplasmic immune reaction in the cell body and processes of astrocytes (Fig. 7c).



Figures. (7a-c): Photomicrographs of GFAP immunostained sections of rat hippocampus (dentate gyrus) of (a): control group showing strong positive cytoplasmic immune reaction in the cell body and processes of astrocytes (arrows). (b): PTU group showing weak immunoreactivity in the astrocytes. (c): PTU+Ash group showing strong positive cytoplasmic immune reaction in the cell body and processes of astrocytes (arrows). (7a-c x200- scale bar 50µm)

Morphometric results

PTU treated group showed significant decrease on epiphyseal growth plate cartilage, area% of PAS reaction in thyroid gland and optical density of PAS reaction in bone as compared to control and therapeutic groups. Therapeutic group revealed marked improvement regarding the aforementioned parameters. In spite of that, there was still significant difference when compared to the control normal (Table 2, 3).

Table 2: Mean thickness (µm) of growth plate cartilage (±SE) in the studied groups

Groups	Mean±SE
Control group	462.3±3.2
PUT group	106.5±2.4*
Ashwagandha+PUT group	336.3±2.8*#

Data were expressed as mean values ± SE with significant difference when P value ≤0.05.
*significant difference as compared with control
significant difference as compared with PUT

Table 3: Mean±SE area% and optical density of PAS reaction in thyroid gland and bone respectively in the studied groups

	Control group	PUT group	Ashwagandha + PUT group
Area% of PAS reaction in thyroid gland	55±3.8	6.6±0.19*	41.8±0.25*#
Optical density of PAS reaction in bone	0.78±0.01	0.52±0.02*	0.77±0.01#

Data were expressed as mean values ± SE with significant difference when P value ≤0.05.
*significant difference as compared with control
significant difference as compared with PUT

Regarding dentate gyrus region of hippocampus, PTU treated rats showed significant increase in cresyl violet stain area % and decrease in GFAP immunostained astrocytes as compared with control and therapeutic groups. Therapeutic group revealed significant increase in GFAP immunoreactivity as compared with the control group (Table 4).

Table 4: Mean±SE area% and optical density of cresyl violet stain and area % of GFAP in dentate gyrus in the studied groups

	Area% of cresyl violet	Optical density of cresyl violet	Area% of GFAP
Control group	9.78±0.2	0.54±0.01	7.3±0.18
PUT group	18.2±0.28*	0.53±0.002	1.8±0.08*
Ashwagandha+PUT group	9.9±0.33#	0.56±0.01	10.1±0.23*#

Data were expressed as mean values ± SE with significant difference when P value ≤0.05.
*significant difference as compared with control
significant difference as compared with PUT

Discussion

Triiodothyronine (T3) and thyroxin (T4) are thyroid hormones that are produced, stored, and released by the thyroid gland, an endocrine gland. These hormones are necessary for cell formation and growth. One of the thyroid conditions is hypothyroidism, which is characterised by a lack of thyroid hormone production as a result of thyroid gland dysfunction that disrupts hormone synthesis and secretion (28, 29).

The current work sought to assess the hormonal and histological alterations in the hypothyroid-model and their impact on the growing bone and dentate gyrus region of the hippocampal brain. To determine whether ashwagandha extract might potentially protect young rats' bones and hippocampus from the effects of hypothyroidism and its related risks.

PTU caused hypothyroidism biochemically, which is indicated in the measured levels of the hormones T3, T4, and TSH. The statistical analysis revealed a considerable drop in the serum T3 and T4 concentrations; also, the TSH showed a significant rise in comparison to the control values. The current findings were in agreement with a number of studies (26, 30). The theory underlying the PTU action mechanism is based on the fact that the thyroid peroxidase enzyme regulates T4 production, which in turn restricts thyroid hormone release and the conversion of T3 and T4 into their active forms in peripheral tissues (31 and 32).

The thyroid gland's histological findings in the current investigation, such as evidence of hyperplasia, cellular cytoplasmic vacuolation, clogged blood capillaries, stratification of the follicular cells, in addition to disturbed and fused thyroid follicles, corroborated the hypothyroid condition. Additionally, both the follicular cells and the interfollicular cells displayed degenerated and vacuolated thyroglobulin masses. Similar findings were reported by Aboul-Foutoh et al. (33) and El-Tantawy and Abozeid (30).

The cellular cytoplasmic vacuolation may be brought on by fluid retention, excessive glandular activity, or hydropic and vacuolar deterioration (34). Furthermore, the observed stratification of the follicular epithelium may be the result of the cells' increased activity brought on by the high levels of the hormone TSH. Other researchers have previously reported similar findings (30, 33, 35). Additionally, it could be explained by the enhanced functional capacity brought on by the elevated substantive demand. Due to the altered endocrine environment and increased functional demand for injury, resting cells are stimulated to enter the cell cycle (G1) and divide, which results in cellular hyperplasia (36).

According to Standring (37), who observed that prolonged high levels of circulating TSH produce follicular cell hyperplasia and increase stromal vascularity, the higher TSH levels in our study's hypothyroidism model were consistent with these findings. Furthermore, Ramsden (38) claimed that a rise in TSH triggers the thyroid to create vascular endothelial growth factor, a strong angiogenic protein that leads to vascularization.

The PTU-treated group in the current study displayed colloidal degeneration and vacuolation with the majority of the thyroid follicles. It is possible that follicular cells increase their activity in taking in and releasing thyroid hormones into the circulation to make up for the increased demand because PTU does not impact the iodinated thyroglobulin already stored in the gland (39).

The current work revealed a significant improvement in biochemical parameters of T3, T4 and TSH levels in the therapeutic group as compared with hypothyroid-model group. Despite that, these hormones did not reach the normal levels when compared with control group. Our results were in agreement with Abdel-Wahhab et al. (40) who revealed that treatment with ashwagandha methanolic extract enhances thyroid hormone production and reduces oxidative stress.

In contrast to the PTU-group, the thyroid's histological structure significantly improved after co-administration of ashwagandha and PTU, according to the current study. It is possible to read this as meaning that the PTU group's hyper-stimulated TSH levels caused the histopathological abnormalities, which were then ameliorated by the TSH drop. A decrease in blood capillary congestion, increased follicular organisation, and a recovery of thyroglobulin in the follicles were the results of these improvements. The proposed findings in this work were in agreement with a prior study published by Abdel-Wahhab et al. (40), who noted that ashwagandha had a positive effect on hypothyroid models with restoration of normal hormone levels. Ashwagandha exhibited an impact similar to that of eltroxin, according to Purohit & Purohit (41). Ashwagandha may be a secure and efficient alternative for restoring normal thyroid hormone levels in individuals with hypothyroidism, according to human study by Sharma et al. (42).

Endochondral ossification is a process that results in the development of long bones and linear growth. Before developing into hypertrophic chondrocytes and ultimately dying through programmed cell death, chondrocyte progenitor cells undergo clonal expansion and multiply. A mineralizing cartilage matrix is secreted during this process, creating the framework for vascular invasion and bone formation. In response to regional mechanical stresses and paracrine signaling cues, newly laid down bone is continuously remodeled by bone resorbing osteoclasts and bone-forming osteoblasts to shape the developing long bone. Later, linear growth takes place in the specialized epiphyseal growth plates located at the proximal and distal extremities of long bones using the same ordered mechanism (43-45).

The epiphyseal development plate is directly sensitive to T3 because reserve and proliferating chondrocytes express thyroid receptors-α1 and β1 (TR- α1 and TR- β1). T3 promotes hypertrophic chondrocyte differentiation and cell volume increase while stimulating clonal expansion of chondrocyte progenitor cells and inhibiting subsequent cell proliferation (45–46). This explained the reduced growth plate thickness in hypothyroid rats.

T3 induces collagen X and alkaline phosphatase expression in primary growth plate chondrocyte cultures, which increases cartilage matrix mineralization. Several paracrine hormones, such as insulin-like growth factor-1 (IGF1), bone morphogenetic proteins (BMPs), and fibroblast growth factors (FGFs), control the rate of chondrocyte proliferation and differentiation during endochondral ossification. This feedback loop's set-point and rate of linear growth are affected by changes in thyroid status in vivo and are controlled by the availability of T3 and local thyroid hormone metabolism (45).

Additionally, aggrecanase-2 (a disintegrin and metalloproteinase with thrombospondin motifs 1, ADAMTS5) and matrix metalloproteinase-13 (MMP13) are collagen-degrading enzymes that are regulated by T3. T3 also controls the expression of growth plate matrix proteoglycans and collagen-degrading enzymes. The expression of genes that regulate chondrocyte maturation and the synthesis, mineralization, and degradation of cartilage matrix is stimulated by thyroid hormone, which is crucial for the coordinated process of endochondral ossification (45, 47, 48). This explained the reduced matrix deposition as evident by decreased optical density of PAS reaction in hypothyroid rats.

In this study endochondral bone formation was impaired severely in hypothyroid rats but appeared nearly normal in hypothyroid animals treated with ashwagandha. Furthermore, growth retardation as evident by decrease growth plate thickness was only evident in hypothyroid rats but not in other groups, supporting the hypothesis that growth plate chondrocytes is exquisitely sensitive

to thyroid hormones in vivo. It was worth mentioning that the ameliorating effect of ashwagandha on increasing thickness of growth plate cartilage and increased deposition of bone matrix as evident by increased area % of PAS reaction may be attributed to improved serum levels of thyroid hormones where chondrocytes are very sensitive to them as aforementioned before.

The growth plate changes in PTU group in the current work were concomitant with a previous study (49) who revealed that the pre and postnatally, carbimazole induced hypothyroidism and its replacement therapy affected the axial and appendicular skeletal growth. Others (50) revealed that hypothyroidism in children causes' growth arrest, delayed bone maturation, and epiphyseal dysgenesis.

For thyroid hormones, the brain is a crucial target (51). The expression of thyroid hormone receptors in the rat brain grows significantly throughout the perinatal period, and the more severe effects of hypothyroidism cause multiple alterations (52). The hippocampus is a priceless, sensitive region of the brain that plays a large role in emotional and motivational processes. Numerous investigations have documented the susceptibility of various hippocampus regions to developmental hypothyroidism (53).

Hypothyroid rat hippocampus dentate gyrus slices stained with H and E revealed granule cell degeneration and vacuolations in the molecular and polymorphic layers. Due to the abundance of thyroid receptors in the hippocampus, it is a particularly sensitive neuronal structure to the effects of thyroid hormones. The continued presence of these receptors in adulthood would suggest that thyroid hormones have a function in the mature brain (54, 55). The same group displayed poorly defined purple Nissl's granules that were exposed by Cresyl violet stain as well as dark-stained, heterochromatic nuclei. According to Ambrogini et al. (56), thyroid hormones encourage cell viability by inhibiting apoptosis. By attaching to its nuclear receptor, THs is known to influence the cell cycle and regulate nuclear genes (57). Lack of THs impacts apoptotic gene expression and controls cell death during neurogenesis. According to certain studies, THs deficiency increases DNA fragmentation, adversely affects the survival of newborn cells, especially immature neurons, and causes newborn cells to postpone neuronal differentiation (58).

The histological changes detected in dentate gyrus in PTU group were in agreement of a previous study (59) which revealed reduced neuronal and astrocytes survival in hippocampus in adult hypothyroid rats.

Astrocytes serve as the primary neuronal guardians in the central nervous system (CNS) and play important roles in synaptic integration, neuron migration and maturation, myelination, ionic control, and neurotransmitter metabolism. The intermediate filaments of the cell cytoskeleton are made up of intermediate filaments such glial fibrillary acidic protein (GFAP). It is found in astrocytes, and its discovery aids in the highly accurate identification of astrocytes (60). In the current study, it was discovered that hypothyroid rats had fewer GFAP immunostained cells. This was in conformity with the findings presented by Mohammed and Ahmed (59). Remaud et al. (61) demonstrated that short-term adult-onset hypothyroidism significantly impairs dendrite arborization of immature neurons in the sub-granular zone of the dentate gyrus. It has been shown that hypothyroidism is associated with impaired myelination, delayed development of the dendritic tree, reduced glial cells, and axo-dendritic synapses.

Thyroid hormones have been shown to influence astrocyte shape, differentiation, and proliferation in vitro, as well as extracellular matrix (ECM) formation and organisation. In the basal forebrain and hippocampus, thyroid hormones control the vimentin-GFAP switch, a sign of astrocyte development, and the radial astrocyte transition in vivo (62). Though supplementing with T3 did not restore either neuronal or astrocyte IF hyperphosphorylation, it is interesting to note that hypothyroidism affected the phosphorylating mechanism connected to the cytoskeleton of both cells (63).

Restoration of granule cells with sizable vesicular nuclei was seen following ashwagandha extract therapy. We also found a small number of cells with unusually shaped nuclei. In addition, most astrocytes had a significant positive cytoplasmic immunoreaction to GFAP. These results showed that morphology had made some progress. El-Hadidy et al. (64) revealed that ashwagandha extract has antioxidant and anti-inflammatory properties that protect against neurotoxicity. They reported also that ashwagandha extract may stop the reduction in cholinergic action by keeping acetylcholinesterase activity at its usual level. As a memory enhancer, ashwagandha may be advised based on the latter effect. In addition to the advantages already described, ashwagandha has a normalizing effect on thyroid hormone levels and its effects on neurons are noteworthy.

Both THs (T4 and T3) can enter the central nervous system (CNS) through certain transporters; T4 is changed into the active TH (T3) in glial cells, astrocytes, and tanycytes, however neurons and developing oligodendrocytes are the primary target cells (65). The migration, development, differentiation, and signalling of brain cells are just a few of the many effects that thyroid hormones have on the CNS (51). The steroid/TH receptor superfamily of ligand-dependent transcription factors includes the nuclear TH receptor. Therefore, thyroid hormones favorably control several genes (66). Through CNS re-myelination and non-genomic and genomic impacts on mitochondrial biosynthesis and function, THs aid in CNS repair. Therefore, poor mitochondrial biogenesis and function in humans are associated with lower TH activity (67–68).

It worth mentioning that recommended dose of ashwagandha extract in human ranges between 250 to 1250mg/day. It was also reported by Raut et al. (69) its efficacy at dose dependent manner and its safety at the same time. So, using higher doses, as equivalent to human, than the used dose in our study may exert better effect near control normal.

Conclusion

Administration of Ashwagandha with PTU displayed protective effect on the thyroid gland and its associated histological changes in growth plate cartilage and dentate gyrus. Higher doses of ashwagandha should be used for extrapolation if it may give better results than the used dose.

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Conflict of Interest

No conflict of interest

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