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Research Article

Effect of Glutamine supplementation on histological and some pathophysiological parameters of the male rat with induced hypothyroidism by propylthiouracil (PTU)

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Abstract

The study was conducted to evaluate the histological and some physiological parameters in male rats with induced hypothyroidism by propylthiouracil (PTU) for 30 and 45 days and the effect of glutamine (Gln) supplementation on thyroid recovery and enhanced its physiological function. The results revealed a highly significant decrease in T4 in rats of the hypothyroid group during 30 and 45 days than the control group (72.23±9.82M mol/L). The treated group with Gln had a significant increase in T4 post 30 and 45 days compared to hypothyroidism and control groups. The results also showed differences in TSH levels in rats as a significant increase in the hypothyroid group for 30 and 45 days with a mean of (15.65±7.01 and 18.85±7.95UUI/ml, respectively). The mean concentration of TSH in rats with hypothyroidism treated with Gln showed a significant decrease compared to the hypothyroidism group.The results also showed a highly significant decrease in iron and ferritin levels in comparison to the control group post 30 and 45 days, with mean ferritin concentrations of 7.56±2.64 and 4.71±1.43ng/ml. The results revealed that iron and Ferritin level of hypothyroid rats treated with Gln was increased compared to the hypothyroidism group. The results of histological examinations in rats with induced hypothyroidism post 30 days showed thyroid follicles with variable size and shapes, lining with simple cuboidal epithelial cells, interfollicular adenomatosis, scanty or mild colloid within follicles lumen, congested blood vessels, inactive follicles have squamous epithelium with vesicular nuclei, vacuolated cytoplasm and moderate depletion of Para follicular cells. Also, severe alternations were observed post 45 days of induction like disintegrated thyroid follicles, shrunken and atrophied, hyperemia, hyperplasia, heavy infiltration of inflammatory cells, and congested large blood vessels. Moreover, the sections of treated with Gln post 30 and 45 days showed regenerated follicles and recovery of normal parenchymal tissue, the follicles grouped as lobules, each follicle lining with simple cuboidal epithelium, and a moderate amount of colloid material.

Keywords: Propylthiouracil, Hypothyroidism, Biochemical parameters, Histology.

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Introduction

The thyroid is the largest vascularized endocrine gland, relatively small and in normal adult weights approximately 10-20g, but it regulates the body's metabolism, growth, development, oxygen consumption, and metabolism of lipids, carbohydrates, protein, nucleic acids, vitamins, and inorganic ions. In addition, thyroid hormones (THs) are used as indicators of thyroid function in human and experimental animals (Delbert 2009; Benvenga et al. 2018). Hypothyroidism is an underactive thyroid gland, which does not secrete enoughTHs and is characterized as a sickness i.e. decline of 1-2% in the synthesis of THs (Gurevitz et al. 2011; Chaulin et al. 2021).

A complex relationship exists between the thyroid and liver in health and disease. The liver plays a vital role in THs activation and inactivation, transport, and metabolism and THs affect the activities of hepatocytes and hepatic metabolism. The serum liver enzyme abnormalities observed in hypothyroidism may be related to impaired lipid metabolism, hepatic steatosis, or hypothyroidism-induced myopathy. Severe hypothyroidism may have biochemical and

clinical features such as hyperammonemia and ascites mimicking liver failure symptoms (Piantanida et al. 2020).

Iron is one of the essential elements for all cells to maintain their metabolic efficacy. It can be converted between two forms of ferrous Fe^{2+} and ferric Fe^{3+}; it also forms the blood Hemoglobin (HB). Therefore, iron deficiency means a lack of HB. It plays a vital role in stimulating the secretory activity of the thyroid gland by affecting the efficacy of the iron-containing thyroid peroxidase enzyme responsible for stimulating the first steps in the production of THs. Thus, hypothyroidism and iron deficiency are highly correlated (Zimmermann 2006). Ferritin is an iron storage protein found in almost all body tissues. Serum ferritin levels have also been altered in patients with thyroid disease, especially hypothyroidism. Hence, changes in the serum concentrations of ferritin reflect thyroid function; there is a correlation between T3 levels and ferritin expression (Akhter et al. 2012). There is a significant negative correlation between TSH and HB. The symptoms of anemia were more in hypothyroid patients than in euthyroid anemic patients (Yadav et al. 2019).

Glutamine is the most abundant free amino acid in the body. It is the principal metabolic source for enterocytes, lymphocytes, macrophages, and fibroblasts. It is considered an essential amino acid under inflammatory conditions such as infection and injury (Cruzat et al. 2018; Li et al. 2019). Therefore, this study evaluated the histological and some physiological parameters of induced hypothyroidism by propylthiouracil (PTU) in the male rat for 30 and 45 days and the effect of glutamine (Gln) supplementation on thyroid recovery and function.

Materials and Methods

Experimental animals: The present study was conducted on healthy adult male Wistar albino rats (*Rattus norvegicus*) aged 10-12 weeks and weighing 200-250g. The animals were obtained from the Medicine College of Basra University and the College of Veterinary Medicine, University of Baghdad. They were transferred to the animal house of the Biology Department and housed under standard conditions with approximately 12h light/12h dark and a temperature of 25±2°C. All rats were fed free access to commercial feed pellets and drinking water *ad libitum* (Gravian et al. 2007). According to the university animal ethics committee, the animal care and experimental ethics standard protocol was followed.

Preparation of propylthiouracil and glutamine: Tablets of propylthiouracil (PTU) and glutamine (Gln) were ground as powder, and doses of each one were weighed daily, i.e. 0.05% of PTU was prepared using 50mg drug dissolved in 1 liter distilled water and added to the drinking water of rats during the experimental period of 30 and 45 days (Gravina et al. 2007; Sewan 2017). Gln was prepared by dissolving 0.25mg of glutamine amino acid in 1ml distilled water (Onaolapo et al. 2013).

Experimental design: A total number of 48 adult male rats (*Rattus norvegicus)* were used in this experiment. They were divided randomly into 3 subgroups, each with 16 rats for each group. These treatments were hypothyroidism, hypothyroidism**+**glutamine, and control group; rats of the control group were given water during experimental periods, while hypothyroidism rats were given 0.5% PTU in their drinking water daily for 30 and 45 days. The third group received daily a dose of 0.5% PTU via drinking water to induce hypothyroidism, and a dose of 0.25g/kg body weight Gln, injected (IP) as 1ml twice / week (Onaolapo et al. 2013; Sewan 2015).

Sacrificed the experimental rats: The experiment was performed for 30 and 45 days, and all animals were weighed on zero days (pretreatment). At the end of the experiment, all rats from each group (control, hypothyroidism, and treated) were sacrificed after being anesthetized with an overdose of chloroform. Before sacrificing, the samples were collected as follows.

Blood collection: After anesthesia, about 4-5ml of blood was collected from their heart by cardiac

Table 1. The mean value of TSH and T4 in all tested groups compared to control post 30 and 45 days of hypothyroidism induction. The value expressed as mean±SD.

Parameters \ Group	T4 (M mol/L) (mean±SD)	TSH (U UI/ml) (mean±SD)
Control group (30) days	67.75±7.62[a]	1.62±1.24[a]
Hypothyroidism group (30) days	9.56±1.25[b]	15.65±7.01[b]
Treated group (30) days	43.35±14.25[b]	7.93±2.14[a]
Control group (45) days	72.23±9.82[a]	2.01±1.11[a]
Hypothyroidism group (45) days	9.06±0.98[b]	18.85±7.95[b]
Treated group (45) days	49.58±6.61[b]	6.08±2.09[a]

Different letters indicate significant differences among groups at ($P \leq 0.05$).

Table 1. Mean concentration of Iron and ferritin levels in all tested groups post 30 and 45 days ofinduction. All values were expressed as mean±SD.

Parameters \ Group	Iron (µg/ml) (mean±SD)	ferritin (µg/ml) (mean±SD)
Control group (30) days	67.37±6.54[a]	81.81±1.68[a]
Hypothyroidism group (30) days	15.11±5.56[b]	7.56±2.64[b]
Treated group (30) days	28.63±4.54[b]	10.8±1.00[b]
Control group (45) days	71.93±8.59[a]	77.87±11.38[a]
Hypothyroidism group (45) days	11.5±2.87[b]	4.71±1.43[b]
Treated group (45) days	31.68±5.4[b]	13.02±1.9[b]

Different letters indicate significant differences among groups at ($P \leq 0.05$).

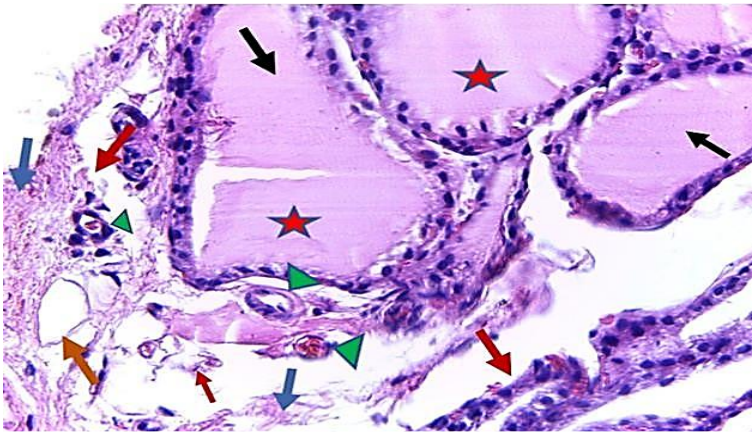


Fig.1. Transverse section in thyroid gland from control rat showed large thyroid follicles (Black darts), fill with colloid (Red stars) and all follicles surrounded with vascular connective tissue (Red darts) with capillaries (Green triangles), collagen fibers and lymphatic vessel (Blue darts). (H&E) stain (X40).

puncture using disposable syringes. The blood was allowed to clot and left at room temperature for 15min. Then 5ml of blood was poured into jelly test tubes free of anticoagulant and centrifuged at 3000rpm for 15min to separate sera that were transferred to the Eppendorf tubes and stored at -20ºC for biochemical tests of liver enzymes (AST and ALT), measuring Iron and Ferritin and hormonal analysis (TSH and T4) (Cray et al. 2009).

Tissue sampling: samples of the thyroid gland from each experimental group were removed after 30, and 45 days, washed with normal saline to removed blood and tissue debris, then cut to a proper size and fixed into 10% neutral formalin solution at room temperature for 24 h. (Luna1968).

Biochemical study: A diagnostic T4 kit (Boditech,

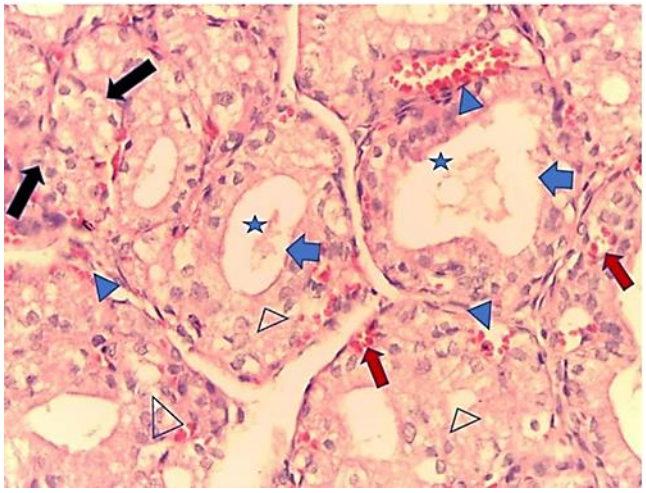


Fig.2. Section in thyroid gland post (30) days of hypothyroidism induction showed interfollicular adenomatosis (Red darts), intrafollicular hyperplasia (Blue darts), hyperplastic changes (Triangles), scanty colloid (Blue stars), hyperemia between thyrocyte (Red darts) and thyroid follicles (Blue triangles) (H&E) stain (X40).

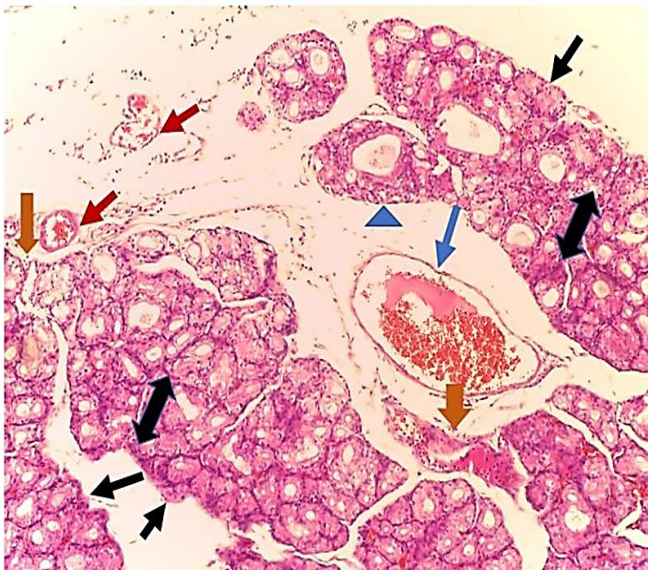
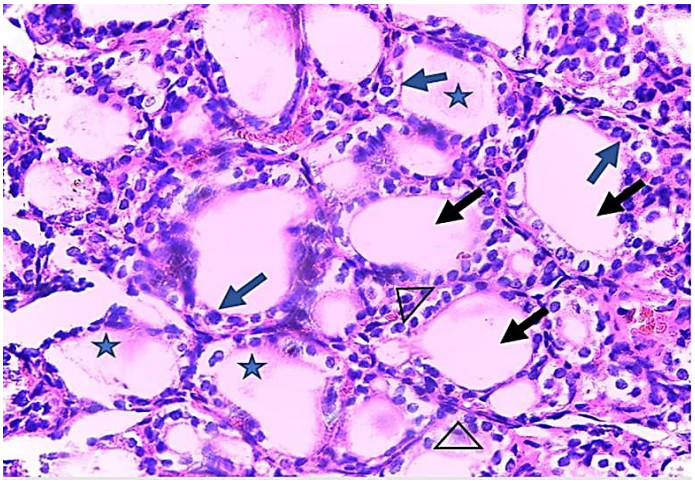


Fig.3. Photograph of thyroid section from hypothyroid rat post (45) days of induction showed atrophied, shrinking follicles (Black darts), hyperplastic capillaries (Two-headed black darts), changes in intrafollicular adenomatosis (Blue triangle), congested large blood vessel (Blue darts), and dense connective tissue (Pink darts) separated the thyroid lobules (H&E) stain (X10).

Korea) was used to measure serum T4 concentration (Wahlin et al. 1998). TSH concentration is regarded as a tool in hypothyroidism diagnosis and was measured by a kit from Boditech, Korea (Tietz et al. 1995). Also, liver enzymes of AST and ALT were measured at 340nm based on Schumann et al. (2010). Estimation of Ferritin was done by a sandwich immune detection method (Knovich et al. 2009) for iron, diagnostic kit of Biolabo was used (Tietz 1999).

Histological study: All samples of the thyroid gland were processed and cut into sections with 7µm thickness and stained using hematoxyline & eosin. Then they were examined and photographed using a Leica digital light microscopic camera type (Leica, Allendale, NJ). Histological changes in the thyroid glandwere evaluated in each group for two periods. To detect alternations such as the presence or absence, and distribution of polysaccharides in the thyroids tissue of control and treated groups, the histological sections were prepared for the



histochemical analysis and stained with Periodic acid Schiff reagent (PAS) (Drury et al. 1967).

Statistical analysis: The results were analyzed with ANOVA in SPSS (2012, statistics version 21. IBM). The data were expressed as mean ± standard deviation (mean±SD). The least significant difference test (LSD) was used to test differences between means of groups at $P \leq 0.05$.

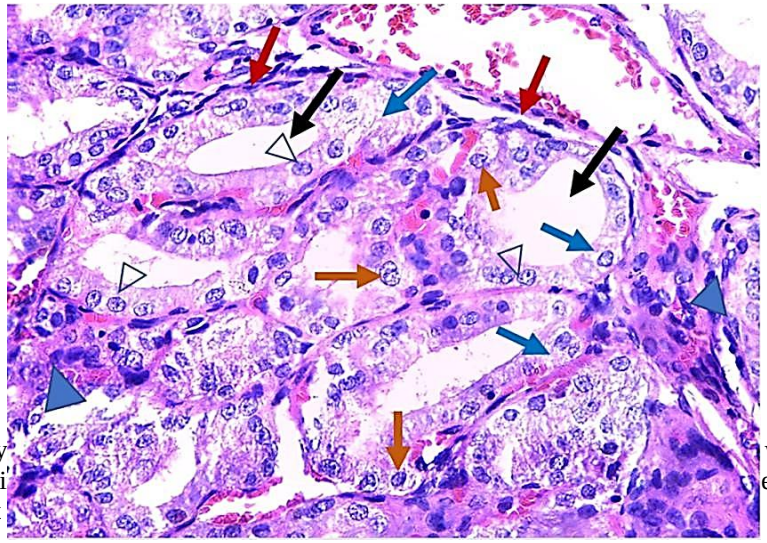


Fig.4. Photomicrograph on thy with (Gln) showed more large regular thyroid (Black darts), li e (Black triangles), some large follicles with partially amount

Results

Determination of TSH and T4: A significant decrease ($P<0.05$) in T4 was found in rats of the hypothyroid group for 30 and 45 days with a mean of 9.56±1.25 and 9.06±0.98Mmol/L, respectively, compared to control group (72.23±9.82M mol/L). The treated group showed a significant increase in the level of T4 ($P<0.05$) after 30 and 45 days with mean concentrations of 43.35±14.25 and 49.58±6.61Mmol/L, respectively, compared to the hypothyroidism and control group (Table 1). Variation in TSH level in all rats revealed a high significant increase ($P<0.05$) in the hypothyroid group in 30 and 45 days with mean values of 15.65±7.01 and 18.85±7.95U UI/ml compared to control one (1.62±1.24U UI/ml). Moreover, the mean concentration of TSH in rats of the

Fig.5. Photomicrograph on thyroid section from hypothyroid rats post 45 days induction and treated with (Gln) showed more large regular thyroid (Black darts), lining with typical cuboid (Blue darts), follicleshyperplastic interfollicular connective (Black triangles), thin septa of collagen fibers (Brown darts) and separated the follicles and cytoplasm (Pink darts) between thyrocytes and follicles (Blue triangles) (H&E) stain.

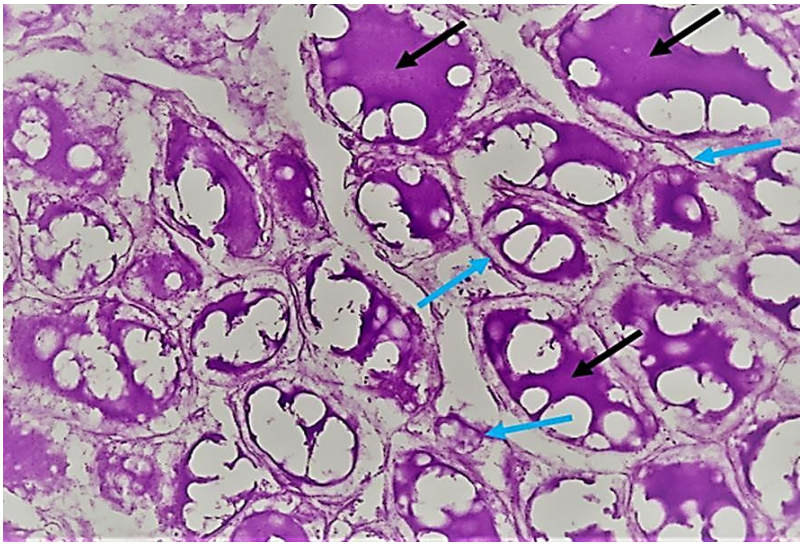


Fig.6. Photomicrograph of a section in the thyroid gland related to control rat showing strong PAS reaction in the colloid (Black darts) and a moderate reaction in the basement membrane (Blue darts) (PAS) stain(x40).

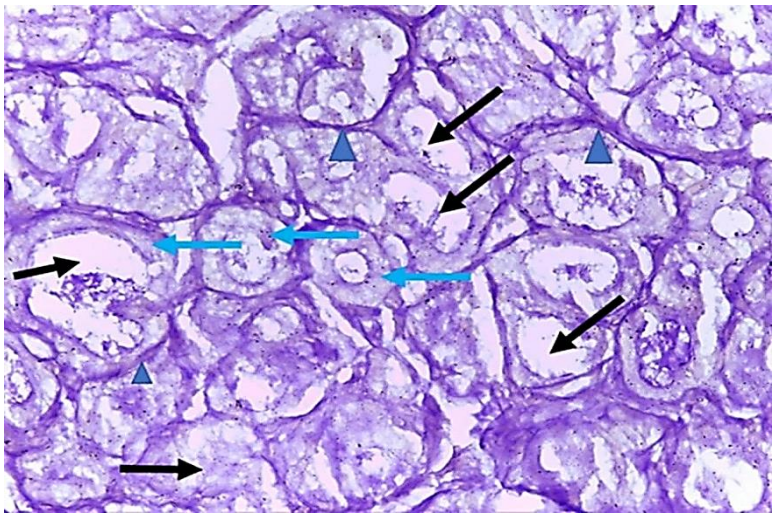


Fig.7. photomicrograph of a section in the thyroid gland post (30) days of induction showing distended, large follicles with weak PAS reaction in colloid (Black darts), the lining epithelial cells not obvious (Blue darts) and moderate PAS reaction in basement membrane (Triangles) (PAS) stain (X40).

hypothyroidism+Gln group showed a significant decrease compared to the hypothyroidism group with mean values of 7.93±2.14 and 6.08±2.09U UI/ml (Table 1).

Determination of Iron and ferritin: The results showed that the rats injected with PTU for 30 and 45 days had a highly significant decrease in serum levels of iron and iron stores (ferritin) (P≤0.05) in comparison to the control group (Table 2). The mean concentration of iron was 15.11±5.56 and 11.50±2.87µg/dl compared to the control group (71.93±8.59µg/dl) after 30 and 45). The mean ferritin concentration was recorded at 7.56±2.64 and 4.71±1.43ng/ml compared to the control (81.81±11.68ng/ml). The results revealed that Gln attenuated serum levels of iron and Ferritin in hypothyroid rats that were more than those of the mean concentration of hypothyroid rats (28.63±4.54 and 31.68±4.50ug/dl) and (10.80±1.00 and 13.02 ±1.90ng/ml), respectively (P≤0.05) (Table 2).

Histological study: Histological sections of the thyroid gland in control rats showed that this gland is

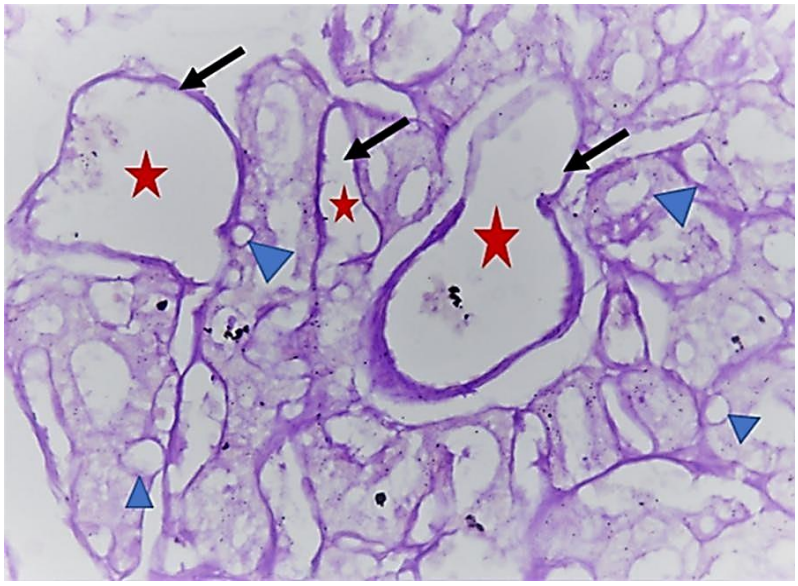


Fig.8. Photomicrograph of a section in the thyroid gland post (45) days of induction showing thyroid follicles with weak PAS reaction (Black darts), the lumen loss colloid completely (Triangles), other few follicles with large vacuoles (Stars) and referred to negative PAS reaction (PAS) stain (X40).

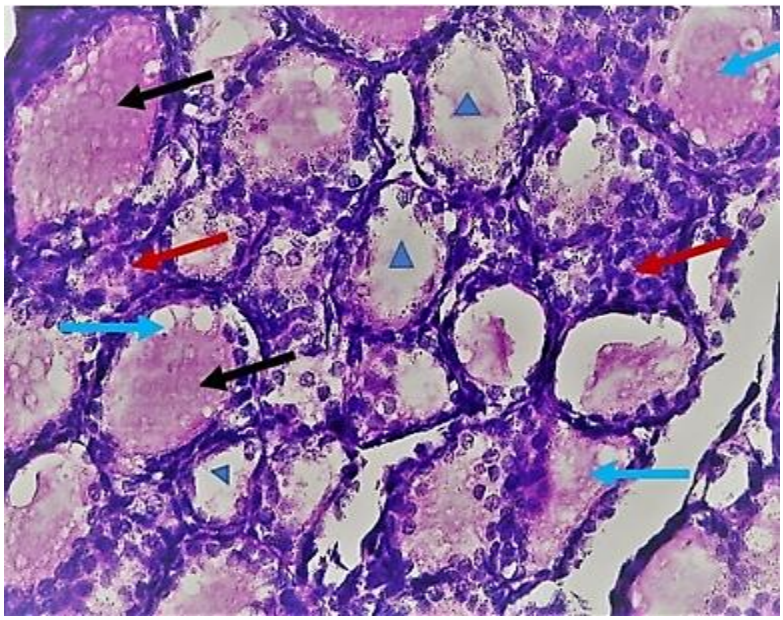


Fig.9. Section in thyroid of hypothyroid rats treated with (Gln) post (30) days showing follicles with strong PAS reaction (Black darts), few follicles empty of (Triangles) others with strong staining but with periphery (Blue darts) and dense positive staining at interfollicular (Brown darts) (X40).

composed of lobules surrounded by capsules, including fibrous connective tissue as thin septa or trabeculae extends deeply to the gland parenchyma dividing the gland into rounded follicles. Each follicle is lined with simple cuboidal epithelium around the lumen filled with colloid (Fig. 1). Thyroid tissue of rats with induced hypothyroidism after 30 days showedthyroid follicles with variable size and shapes, lining with low simple cuboidal epithelial cells. Some follicles are lined with columnar cells or those with less activity or inactive have squamous epithelium with vacuolated cytoplasm, large hypo chromatic nuclei, moderate depletion of Para follicular cells among the follicles, infiltration of

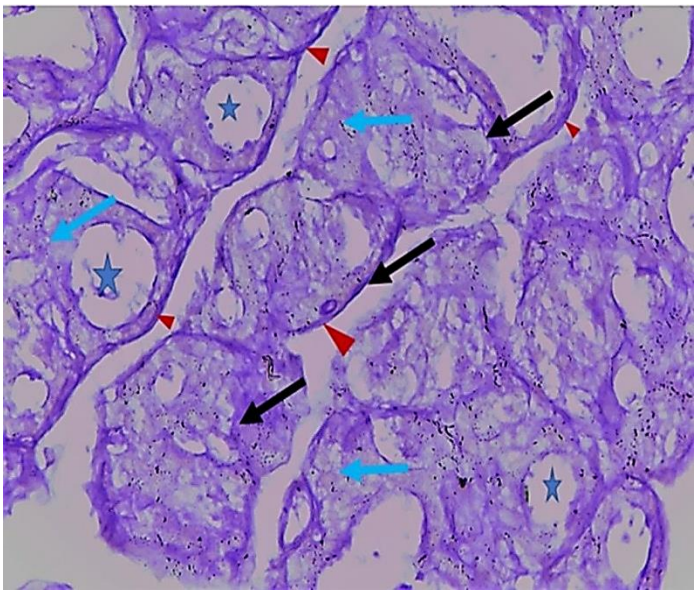


Fig.10. Section in thyroid of hypothyroid rats treated with (Gln) post (30) days showing reaction (Black darts), few follicles empty of colloid (Blue Stars), others with strong staining but with vacuolated colloid at (Brown Stars) but and dense positive staining (Blue darts) (X40).

inflammatory cells. The focal inflammation was observed in scanty colloid material in the large distended follicles. Also, pools of follicular cells without lumen form interfollicular adenomatosis follicles, congested capillaries, large blood vessels, veins, and vascularized connective tissue (Fig. 2). The results showed that the histological alternations of thyroid glands related to hypothyroid after 45 days revealed disintegrated thyroid follicles, most shrunken and atrophied, hyperemia between lining epithelium, other follicles showed exfoliated cells in lumen, degenerated follicular epithelium, hyperplasia and squamous metaplasia of lining epithelium, severe hemorrhage. Moreover, the follicles lumens showed empty of colloid or with scanty material and dense connective tissue extending and separating the follicles (Fig. 3).

Histological observations on thyroid sections of the hypothyroidism rats after 30 days treated with Gln showed more regenerated follicles and recovery of normal parenchyma tissues. The follicles exhibited normal shape, each lining with simple cuboidal epithelium. The follicular cell with an obvious dark oval nucleus, mild vacuolated cytoplasm, Para follicular cells among the follicles, moderate colloid material,and mild hyperemia (Fig. 4). Post 45 days of treatment with Gln, thyroid tissue revealed regenerated rounded follicles lining with normal follicular cells (thyrocytes) with a dark nucleus, an increase in the height of the follicular cell, less congested blood vessels, large follicles with empty lumen found peripherally, mild inflammatory cells, complete recovery of thyroid parenchymal, and the tissue similar to control group (Fig. 5). Periodic acid Schiff (PAS) variable changes in the thyroid tissue; the control group showed rounded thyroid follicles, lined by cell epithelium with squamous or cubic shape, where nuclei cannot be seen due to the stain. The cytoplasm of follicular cells showed a strong positive reaction with PAS, and the parafollicular cells. The colloid inside the follicle lumen was strongly marked with homogenous distribution and moderate PAS reaction in the basement membrane, indicating a high concentration of polysaccharides (Fig. 6). Histological observations in rats treated with PTU after 30 days of hypothyroidism showed detached epithelial cells, mild to moderate reactionwith colloid within follicles lumen and other follicles without a lumen or showed weak reaction and moderate (PAS) reaction of basal lamina (Fig. 7). More severe

changes in the thyroid gland post 45 days of hypothyroidism induction were observed, including the destruction of most thyroid follicles, empty lumen, and scanty or mild colloid material in thyroid follicles, the (PAS) stain referred to weak positive staining in colloid and only moderate PAS reactionin the basement membrane of large follicles while other involute follicles showed weak PAS reaction (Fig. 8).

The results showed the role of Gln in the synthesis and secretion of colloid, based on the results of hypothyroid rats treated with Gln for 30 days of induction as normally rounded folliclesand a variable number of vacuolated colloid within the follicle lumen. A marked increase of PAS-positive reaction was observed in colloid and within interfollicular connective tissue, although few follicles still with scanty colloid (Fig. 9). The results of hypothyroid rats post 45days of induction and treated with Gln showed larger follicles with colloid and an increase of PAS-positive reaction in homogenous colloid, and a strong reaction with PAS in the basement membrane nevertheless some follicles were empty of colloid and some follicles contain peripheral vacuoles (Fig. 10).

Discussion

The results of the present study showed that hypothyroidism causes a significant increase ($P \leq 0.05$) in TSH levels, while the control group showed a gradual decrease in its concentrationthan the induced groups at 30 and 45 days. This may be due to hypothyroidism, in which the thyroid produces insufficient T3 and T4. Also, TSH values had a higher range in hypothyroidism may be corrected by Gln amino acid. The results agreed with other studies that deficiency of THs is a feature of the clinical syndrome of hypothyroidism, leading to diminishing all metabolic processes marked by elevated TSH and reduced T3 and T4 (Stephenetal 2012). The inhibition of these enzymes results in decreased levels of circulating thyroid hormones that lead to increased secretion of TSH by providing a growth stimulus to the thyroid, which agrees with the findings of Udgata & Naik (2007). The effect on the thyroid gland by PTU may be attributed to the inhibition of thyroid peroxidase and 5-deiodinase, which are known as key enzymes involved in thyroid hormone biosynthesis (Sewan et al. 2017).

Iron deficiency anemia (IDA) is a frequent clinical condition accompanying thyroid dysfunction, including hypothyroidism. The THs can directly affect blood formation, especially red blood cells, by enhancing erythropoietin production, which is necessary for erythrocyte proliferation (Bashboosh 2017). All body cells contain ferritin, which acts as a reserve of iron, and small amounts are secreted into the serum to form hemoglobin and other heme proteins (Winter et al. 2014). Ferritins contribute to normal iron flow, maintenance of iron concentrates for iron cofactor syntheses, sequestration of iron from invading pathogens, oxidant protection, and oxidative stress recovery (Theil 2013). The mechanism by which Fe status influences thyroid and iodine metabolism are still unclear. IDA could impair thyroid metabolism through anemia and lowered oxygen transport. This may alter thyroid metabolism's central nervous system control (Starchl et al. 2021) and nuclear T3 binding. In summary, IDA decreases serum T4 and T3 concentrations, reduces the peripheral conversion of T4 to T3, decreases T3 metabolism (turn over), decreases hepatic T4-5'-deiodinase, and may increase circulating (TSH) activities (Khaleghnia et al. 2021).

Ferritin showed a gradual increase with a significant difference in all rats treated with Gln compared to the hypothyroidism group. This may be related to the effect of PTU metabolites and the disturbances with THs which affect blood cell formation and hemoglobin synthesis. It affects on nutrition and metabolism of protein or diminishes iron. These results agree with a recent study that revealed serum ferritin is an iron storage protein present in almost all cells, and the altered level of serum ferritin has been reportedin all patients with thyroid disease (Sahana & Kruthi 2020).

In the current study, the histological examinations of the thyroid gland showed hypothyroid statuses

such as hyperplasia signs, cellular cytoplasmic vacuolation, dilated congested blood capillaries, increase in the height of the follicular cells, and the increase in the diameters of the thyroid follicles. In addition, degenerated and vacuolated thyroglobulin masses were shown in the follicularand interfollicular cells. Similar results were reported by Underwood & Cross (2009). Also, PTU inducing hypothyroidism was reported by Woeber (2000) to cause changes in other organs, such as oxidative stress in the cerebellum of the experimental rat brain that lead to thyroid tissues damage and apoptosis of thyroid follicles. Moreover, post 30 and 45 days of induction with PAS stain, hypothyroid rats showed weak positive staining in colloid and only moderated PAS reaction in the basement membrane of large follicles. Other involute follicles showed weak PAS reaction and mild and scanty homogenous colloid within the lumen of the follicle. This revealed thyroid gland dysfunction, less TG secretion, an inactive storage form of thyroid hormones. This colloid is thyroglobulin, an acidophilic secretory glycoprotein that is PAS-positive. The colloid is an acidophilic secretory glycoprotein that is PAS-positive. Thus under the influence of the TSH, the follicular cells become tall and columnar, demonstrating the heavy activity of the follicular cells. The TSH also enhances the exocytosis, synthesis, and iodination of TG. It also increases endocytosis and intracellular breakdown of colloid substance, thus, the intraluminal colloid is significantly reduced, which is explained by thyroid gland enlargement (Yildirim et al. 2017). While sections of the thyroid gland of hypothyroid rats treated with Gln were small, fragmented, and clumped (TG) within the intrafollicular colloid surrounded by a highcuboidal epithelium. This result is in agreement with other study showed the role of Gln that amino acids can be obtained from endogenous and exogenous proteins. Their availability is important for cell survival, maintenance, and proliferation. Gln is the most abundant and versatile amino acid in the body. It is important for intermediary metabolism, inter-organ nitrogen exchange through ammonia (NH_3) transport between tissues, and pH homeostasis (Cruzat et al. 2018).

Conclusions

This study demonstrated the effect of PTU as anti-thyroid drug on thyroid tissue and the role of glutamine (Gln) as a supplement to inhibit the side effect of PTU having the ability to regenerate its normal structure of thyroid follicles and enhance the synthesis and deposit of TG within follicles lumen. The results also showed a close relationship between hypothyroidism and anemia caused by thyroid gland dysfunction and decreased iron and ferritin storage. The study proved the relationship between TSH and T4, and hypothyroidism. This is related to thyroid gland dysfunction or unreactive. The study also showed the importance of Gln as a nutrient supplement for hypothyroidism disorder, especially iron levels and ferritin concentration gradually returning to the nearest normal values.

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