Original Article
Gastric Mucosa Damage and Impairment of Secondary Immune Response in Dysthyroidism is Associated with TNF-α Expression.

Keywords: Ulceration, oxidative stress, immunoglobulin-G, inflammation

Aim: This study aims to determine the relationship between immune response in rats with altered thyroid state and expression of TNF-α in gastric tissue. Methods: Thirty male wistar rats were randomly grouped into three; Control, hypothyroid and hyperthyroid. Hypothyroidism and hyperthyroidism were induced by the administration of carbimazole and levothyroxine respectively. Serum TNF-α and immunoglobulin G (IgG) were determined as markers of inflammation and secondary immune response respectively. Indices of lipid peroxidation were also evaluated in gastric tissue while sections of gastric tissue were taken for histological evaluation. Results: Hyperthyroid group had a significant decrease in gastric IgG compared with control (P<0.01), while there was no significant difference between the gastric IgG of control and hypothyroid. Hypothyroid and hyperthyroid groups had significant increase in gastric TNF-α compared with control (P<0.01), but in the serum only the hypothyroid group increased significantly (P<0.01). The serum concentrations of IgG and TNF-α of all groups were significantly higher compared with that of the stomach tissues. Hypothyroid and hyperthyroid groups also had significantly raised MDA, but decreased levels of SOD and catalase compared with controls in gastric tissue. Altered thyroid state led to mild inflammation and ulceration of gastric mucosa. Conclusion: Dyshyroidism leads to impairment of secondary immune response resulting in gastric mucosa damage, which is associated with TNF-α expression and oxidative stress. However, the TNF-α and IgG were markedly expressed in the serum than gastric tissues.

Introduction

Thyroid hormones act on almost all organs throughout the body, including the gut and visceral, therefore disturbances in thyroid function may have gastrointestinal manifestations [1]. Basal metabolism and oxidative processes are regulated by thyroid hormones [2]. The hormones act by accelerating basal metabolism, thus increasing rates of both catabolic and anabolic reactions. They modulate the number and activity of mitochondrial respiratory chain components, accelerating oxygen (O2) consumption, leading to an enhanced generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS) within the target tissues [3]. These result in the consumption of cellular antioxidants and inactivation of antioxidant enzymes, thus inducing oxidative stress [4-5, 6].

These ROS have a high reactivity potential, thus causing cytotoxicity and oxidative damage of cellular macromolecules like proteins, lipids and DNA [7]. The host cells are protected from these harmful effects through the antioxidant system which scavenges produced free radicals. Among the enzymatic antioxidants, are glutathione reductase (GR), glutathione peroxidase (GPx), catalase (CAT), superoxide dismutase (SOD), on the other hand the non-enzymatic antioxidants are vitamin E, vitamin C, β-carotene, flavonoids, and glutathione (GSH) [8]. Oxidative stress occurs when ROS generation exceeds the antioxidant capacity of cells. A compromise in this scavenging mechanism leads to the occurrence of toxicity in biomembranes, and therefore lipid peroxidation with consequent production of malondialdehyde (MDA) [9]. Reactive Oxygen Species have been implicated in a number of disease conditions such as Alzheimer’s disease, Huntington’s disease and many other neurodegenerative conditions [10].

Previous studies have reported an increased generation of ROS in hyperthyroidism and hypothyroidism [11-12, 13], with a resultant increase in lipid peroxidation [14, 15]. Reduced activities of...
antioxidant defence system enzymes have been reported in hyperthyroid as well as hypothyroid states [14, 16-17].

The reactive oxygen species mediate signal transduction pathway which induces production of cytokines from different cells [18, 19]. Previous studies showed that antioxidants inhibit the activation of oxidant-mediated transcription factors and potentially prevent the transcriptional activation of inflammatory cytokines [20, 21]. TNF-α a cytokine which is produced by activated macrophages, T-lymphocytes, and natural killer (NK) cells is an important mediator of inflammatory and immune functions which is linked to the pathogenesis of inflammatory and autoimmune diseases like Graves disease [22].

The gastrointestinal tract which is always in contact with the outside environment is mediated by resident immune cells, intestinal and dietary factors thus it is prone to ROS attack too [23]. The IgG is a type of antibody found in blood and extracellular fluid thus permitting its’ control of body tissue infections [24]. The gut which is the largest lymphoid organ in the body contains majorly lymphocyte and produces a large amount of immunoglobulin (Ig). The mucosal immune system is regulated in a special way to control its constant exposure to viruses, parasites, and bacterial antigens, which are in close contact with a large reservoir of lymphocytes, macrophages, and dendritic cells [24]. The mucosal immune system response is either of suppression or tolerance not like the systemic immune system. Dysfunction of its’ regulatory mechanisms in maintaining a balance between active immunity and tolerance within the gut may lead to mucosal inflammation, mucosal damage and GI diseases [25].

Earlier study by Olaleye et al. [26] reported the importance of thyroid hormone in sustaining gastrointestinal tract integrity, in which thyroxine accelerated gastric ulcer healing by accelerating inflammatory and proliferative phases of healing, with thyroidec- tomy delaying these processes. Therefore, thyroid dysfunction affects all levels of gastrointestinal functions with resultant changes having clinical and biochemical relevance. Thus, investigations on the relationship between the thyroid hormones and the stomach may impact on the diagnosis of gastrointestinal disorders and understanding of its pathophysiology. This study was then designed to investigate the possible relationship between immune response in rats having altered thyroid state and the expression of TNF-α within the gastric tissue.

**MATERIALS AND METHODS**

**Animals**

Thirty (30) male rats weighing between 180 – 220 grams were obtained from the Animal House of the Department of Physiology, Ladoke Akintola University of Technology, Ogbomoso. They were certified healthy as none showed any sign of stress or infection. During the study, the animals were kept in wire mesh cages with ad libitum access to rat pellet and water. The laboratory condition was about 25 - 30°C of temperature and relative humidity of 40–60%, the animals were exposed to 12:12 light cycle during the experiment. A seven (7) days period of acclimatization before the commencement of the experiment was observed.

**Treatments**

Rats were randomly allocated into three groups (n = 10): control, hypothyroid, and hyperthyroid. Hypothyroidism was induced by administration of 5mg/250 g body weight of carbimazole, while hyperthyroidism was induced by administration of 5μg/100 g body weight of levothyroxine as previously described [27, 28]. Treatments lasted for 35 days. The experimental study was in accordance with the Institution’s guidelines and criteria for humane care outlined in the National Health Guidelines for the care and use of Laboratory Animals. Ethical approval for the implementation of the research Clearance was obtained from the Oyo state research ethical review committee, Ministry of Health secretariat Ibadan, Nigeria. The reference number of the ethical approval for this research is AD13/479/143.

**Specimen collection**

At the end of the experimental period, all rats were sacrificed and blood samples were collected by cardiac puncture into a plain bottle. Serum was obtained from the blood after centrifugation for 5 minutes at 3,500 rpm. The serum was used to assay for serum thyroxine stimulating hormone (TSH), Immunoglobin G (IgG) and TNF-α. Stomach from each animal was removed, washed three times with ice-cold saline, blotted on ash-free filter paper and used for the estimation of tissue IgG, TNF-α, MDA, SOD, and catalase.

**Preparation of tissue homogenates**

One part of the stomach was weighed and homogenized in phosphate-buffered saline (PBS) 50mM pH 7.4 for estimation of protein content, catalase and SOD activities, as well as IgG, TNF-α and MDA concentrations. The crude tissue homogenate was centrifuged at 10,000 rpm for 15 minutes at 4°C and the resulting supernatant was used for the various examinations. Protein content in tissue homogenate was measured using Buret’s method while activities of SOD, catalase, and concentration of MDA were determined in tissue homogenates as previously documented [29, 30].

**Estimation of TSH, IgG and TNF-α concentration**

Serum and tissue concentrations of TSH, IgG and TNF-α were measured using ELISA kits manufactured by Elabscience Biotechnology Co. Ltd, Wuhan, China. The assays were carried out following manufacturer’s instructions.

**Principle of ELISA**

In this assay, the antigen - TSH, IgG or TNF-α present in each sample reacts with the corresponding antibody adsorbed to the surface of solid-phase polystyrene microtitre wells. On removal of unbound proteins by washing, the antibodies conjugated with horseradish peroxidase (HRP) form complexes with the previously bound antigen following the addition of a chromogenic substrate, 3, 3, 5, 5-tetramethylbenzidine (TMB). The absorbance at 450 nm is a measure of the concentration of the ‘antigen’ in the test sample.

**ELISA Procedure**

Standard samples were prepared from the calibrator by serial dilution of the calibrator with the diluent concentrate as instructed by the manufacturer. One hundred microliters of the standards and sera/tissue homogenate samples were pipetted into microwells
already coated with specific antibodies and incubated at 37°C for 90 min; the plate was covered during incubation.

Following incubation, the wells were aspirated of their contents without washing and 100 μl of biotinylated detection antibody was added to each well and incubated for 60 minutes at 37°C, after which each well was completely filled with appropriate wash solution. The plate was washed three times.

One hundred microliters of appropriately diluted enzyme–antibody conjugate was pipetted into each well and the plate was incubated at 37°C for 30 minutes. After incubation, another process of washing was performed as described above and 90 μl of TMB substrate solution was added to each well. This was followed by incubation for 15 minutes at 37°C after which 50 μl of stop solution was added to each well. The absorbance (at 450 nm) was determined using ELISA reader.

Histological analysis

Histological studies were done as previously documented [31, 32]. A portion of the stomach was fixed in 10% formal saline for histological examination. The tissues were processed and embedded in paraffin wax. 5μm thick sections were obtained and stained by haematoxylin and eosin (H & E) method and examined under a light microscope to determine morphological changes.

Statistical analysis

Data are expressed as Mean ± Standard deviation. Data were analyzed using one-way analysis of variance complemented with unpaired t-test. Turkey’s Multiple Comparison Test was used as post hoc test, Value of P < 0.01 was considered significant.

RESULTS

Carbimazole treatment led to significant (P < 0.01) increase in serum TSH (60.7 ± 3.4ng/dL) when compared with the control (49.0 ± 7.6ng/dL), while, levothyroxine caused a significant (P < 0.01) decrease in serum TSH (25.4 ± 2.1ng/dL) when compared with control (49.0 ± 7.6ng/dL).

Carbimazole and levothyroxine treatments in separate groups caused significant (P<0.01) decrease in serum IgG as well as increase in stomach tissue TNF-α compared with control, while levothyroxine significantly decreased stomach tissue IgG (P<0.01). In contrary, carbimazole treatment significantly increased serum TNF-α compared with control (P<0.01). However, carbimazole and levothyroxine seems to have no significant effect on stomach IgG and serum TNF-α respectively compared with control (figure 1).

Levothyroxine treatment caused a significant decrease in gastric IgG compared with control (P<0.01), while there was no significant (P >0.01) difference between the gastric IgG of control and hypothyroid. There were significant (P<0.01) differences between the three groups for serum IgG however, the concentrations (IgG) were higher in serum compared with stomach tissue for all the groups. Carbimazole and levothyroxine treatments caused significant increase in gastric TNF-α compared with control (P<0.01), but in the serum only the hypothyroid group increased significantly (P<0.01). It is of note that the serum concentrations of TNF-α of the three groups were significantly higher compared with that of the stomach tissue homogenate (Figure 1).

Carbimazole and levothyroxine in the different groups significantly increased stomach tissue MDA compared with control while decreasing stomach tissue SOD and catalase compared with controls (P<0.01) (figure 2).

Carbimazole treatment caused mild inflammation and mild ulceration in gastric mucosa of treated rats, while levothyroxine treatment caused mild ulceration as well as mild inflammation in gastric mucosa of treated rats (figure 3). The unexposed group showed normal morphology of gastric mucosa (figure 3).

Figure 1: Markers of inflammation in serum and stomach tissue in the different treatment groups.

* = Significant difference between carbimazole treatment and levothyroxine treatment compared with control
** = Significant difference between carbimazole treatment compared with control
***= Significant difference between levothyroxine treatment compared with control
Figure 3: Histology of gastric mucosa (H&E X40).

a.) Normal gastric mucosa in control group.

b.) Mild ulceration in carbimazole treated group.

c.) Mild inflammation in carbimazole treated group.

d.) Mild ulceration in levothyroxine treated group.

e.) Moderate inflammation in levothyroxine treated group.

DISCUSSION

Thyroid hormones modulate the functions of the body cells, tissues, and organs, thus playing essential roles in growth, differentiation, maturation, and metabolism [33]. Hyperthyroidism and hypothyroidism are related to oxidative stress and cellular damage [34]. Thyroid hormones and immune system interaction have also been established from amphibious animals to mammals in their development and function [35, 36]. Mostly, TNF-α is an important mediator of inflammatory and immune functions, therefore it is imperative to study the link between inflammatory and immune responses during thyroid dysfunction. This study evaluated the effect of experimentally altered thyroid state on inflammatory response and oxidative stress as related to gastric mucosa secondary immune response. Treatment with carbimazole and levothyroxine led to hypothyroid and hyperthyroid states, respectively. This was confirmed by measuring serum TSH concentration. Carbimazole caused notable increases in serum TSH when compared with control, confirming hypothyroid state while levothyroxine drastically reduced serum TSH when compared with control, confirming hyperthyroid state. Production of TSH is stimulated by thyrotropin-releasing hormone (TRH) which is secreted in the hypothalamus. This TSH is important in the production and release of T4 and T3 from the thyroid with the release of T4 and T3 exerting a negative feedback mechanism on TSH production [37]. The increased serum TSH has been seen in carbimazole treatment and the decreased serum TSH observed in levothyroxine treatment are due to the negative feedback mechanism along the hypothalamic-pituitary-thyroid axis [38]. The result of this study on TSH conforms with previous studies in which administration of carbimazole increased TSH level and that of levothyroxine decrease TSH level [27-28, 39-40].

Thyroid hormones are important in regulating oxidative metabolism and production of free radicals [41]. They also regulate the synthesis and degradation of enzymes, such as SOD, CAT, Glutathione peroxidase (GPx) and Glutathione reductase; and nonenzymatic antioxidants, including vitamins E & C, glutathione, uric acid, ferritin, transferrin, and ceruloplasmin. Hypermetabolism has been reported to occur as a result of thyroid hormone administration to experimental animals [42]. This hypermetabolic state is accompanied by oxidative stress in several target tissues due to increased formation of free radicals in mitochondria [43, 44]. Oxidative stress is described as an imbalance in the amount of pro-oxidant and antioxidant generation, favouring pro-oxidant generation, antioxidant depletion or both. Hypothyroidism is however associated with lower metabolic rate and a decreased free radical production is therefore expected. Interestingly, many studies found that hypothyroidism is associated with oxidative stress [45, 46] due to insufficient antioxidant production [47]. These ROS are particularly harmful in that they initiate free radical chain reactions that lead to oxidative damage to membrane lipids and other cellular components [48-49, 50]. Furthermore, Venditti et al. [48] attributed the oxidative stress to decreased antioxidant capacity. This reduction in antioxidant capacity exacerbates the oxidative stress.

Malondialdehyde (MDA), a major product of lipid peroxidation, is an indicator of mucosal injury by ROS [51], in this study, tissue MDA was an indicator for pro-oxidant while SOD and Catalase were antioxidant indicators. The results of this study showed that altered thyroid state was accompanied by increased MDA level, while the level of SOD and catalase decreased, corroborating previous studies [43-46, 49-50]. The mechanism of increased oxidative stress in hypothyroidism is attributed to insufficient antioxidant defence system because thyroid hormones are important in the synthesis and degradation of antioxidant enzymes. In the chronic state of hypothyroidism, there is impairment of the redox potential leading to free radical chain reactions and metabolic suppression of antioxidant capacity. The oxidative stress may be due to increasing free radical production which was not compensated by insufficient antioxidants [47]. This hypothyroid induced oxidative stress was also found in the liver, heart and skeletal muscles with consequent lipid peroxidative response in previous finding [52].

Tissue necrosis factor alpha (TNF-α) is a cytokine with various biological effects in many pathophysiological processes [22], having an effect on monocytes, macrophages, lymphocytes and eosinophils. It also increases polymorphonuclear leucocytes adhesion to vascular endothelium, and then increases the phagocytosis, survival at the site of inflammation and deregulation of polymorphonuclear cells which mediate the generation of ROS and their metabolites [53]. The increase in respiratory burst activity of gastric mucosa cells increases the expression of redox-sensitive genes, including those coding for cytokines. This oxidative stress markedly increases the production of TNF-α by gastric cells, hence its release into the circulation [54]. This explains the increased TNF-α in the stomach and serum of hyperthyroid- and hypothyroid-rats, and the higher concentration observed in the serum compared with the gastric tissue. Previous work has supported an association between thyroid hormone-induced oxidative stress and cytokine production [55]. Circulating cytokine level might be different from those in the tissue, although, local cytokines response in Helicobacter pylori-infected subject, as well as the serum and gastric fluid levels of cytokines in gastric diseases infected with Helicobacter pylori have been investigated [56, 57], but no study have compared the serum and stomach tissue cytokine level in altered thyroid state like this study.
Furthermore, total IgG is the most abundant immunoglobulin of internal body fluids and is associated with the secondary immune response. Our finding of reduced total IgG in altered thyroid states corroborates the findings of Singer et al. [58] who observed a suppressed IgG response associated with oxidative stress. This might be responsible for the immunosuppression associated with dysthyroidism. Albeit, the IgG in the stomach tissue was lower in value for all groups compared to what was obtained in the serum. Several investigations have shown inconsistent results on the relationship between thyroid hormone and immune response. In some animal models, increased immune response by thyroid hormones [59], and reduced immune response in thyroidectomy [60]. In other reports thyroid hormones had no effect on immunity [61], and in another study, immunity was suppressed by thyroid hormones [62]. Yao et al. [63] reported an elevation of antibody response with anti-thyroid drug administration. The results of the present study regarding suppression of IgG in response to altered thyroid state particularly in hyperthyroidism with consequent inflammation are consistent with results reported by Gill et al. [64] in which administration of thyroxine to suckling rats induced a precocious decline of both IgG binding and the receptor expression. The result of this study shows that altered thyroid state induced oxidative stress impaired the immune system and the expression of TNF-α is involved in the altered thyroid state induced oxidative stress.

The histomorphological study revealed mild inflammation and ulceration in the gastric mucosa of hypothyroid rats while hyperthyroidism also caused mild ulceration as well as mild inflammation. This implies that the inflammation is common to both hyperthyroidism and hypothyroidism thus corroborate the earlier results obtained for oxidative stress, TNF-alpha, and IgG.

In conclusion, our findings demonstrated that dysthyroidism is associated with oxidative stress and inflammatory response in the gastric mucosa with possible impairment of secondary immune response which is linked to the expression of TNF-α. Therefore, possible studies to explore the clinical impact of oxidative stress in the prognosis of dysthyroidism, the response to therapy and in the possible use of antioxidant therapies as modifiers is recommended.

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Authors’ Information:

Conflicts of Interest:

The authors declare that there are no conflicts of interest.

References


