Effect of Experimentally Induced Hypothyroidism on the Parotid Gland of Adult Male Albino Rats and the possible Role of Thyroid Hormone Supplementation

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Abstract
Thyroid hormones; T3 and T4 are essential for physiological functions of almost all body tissues. The current study was carried out to investigate the effect of hypothyroidism on the histological structure of parotid salivary gland of the rat. This study was conducted using 30 male albino rats. They were divided into three groups, 10 rats each: group 1 (control group), group 2 (hypothyroid group) which received carbimazole (NeoMercazol); antithyroid drug; at a dose of 0.05 mg/day for 3 weeks, and group 3 (thyroid hormone-supplemented animals). At the end of the experiment, the animals were sacrificed. Parotid specimens from all groups were processed for light and electron microscopic studies. Biochemical analysis was carried out to measure the serum levels of triiodothyronine, T4 and TSH. Statistical analysis was carried out. Hypothyroid acini had irregular outlines and were widely separated. Congested blood vessels and cellular infiltration were seen in the interstitial space. The interlobular ducts appeared dilated. Mallory's trichrome stained sections clarified extensive collagen fibers in parotid gland. Immunohistochemical stained sections of hypothyroid group showed weak immune reaction for bcl2 in the cytoplasm of parotid cells. Ultrastructural alterations of parotid cells in hypothyroid group were observed. Some acinar cells showed variable sized vacuoles in their cytoplasm. The parotid gland of thyroid supplemented group still contained distorted acinar cells and did not return to its normal histological structure. In conclusion. The level of T3, T4 decreased and that of TSH increased in the experimental group when compared with control group. Also, there were changes in the histological structure of the parotid salivary gland of the teated group thus, it is recommended to early diagnosis of hypothridism with rapid interference.

Keywords: Thyroid hormones; rat; apoptosis; carbimazole; Bcl2

Introduction
Thyroid hormones; T3 and T4 are essential for physiological functions of almost all body tissues. They regulate reproductive functions, heart rate, body temperature, gastrointestinal motility and emotional stability in addition, they control metabolism of proteins, lipid and carbohydrate. Disruptions of thyroid function may produce various subclinical or clinical manifestations (Shady and Noor El-Deen, 2010). In human, hypothyroidism is one of the most common thyroid disorders (Čakić-Milošević et al., 2004), that may be congenital or acquired (Porth et al., 2004). It may result from dysfunction of thyroid gland itself, impairment in mechanisms which control thyroid hormones formation, or as a complication during treatment of hyperthyroidism. Hypothyroidism is characterized by low metabolic rate resulting in adverse effect on many organs (Khalawi et al., 2013). Its clinical manifestations varies from pale cool puffy skin, dry brittle hair, nails, eyelids dropping, per orbital edema, lethargy and muscle stiffness. Most of hypothyroid patients suffer from decreased gastro intestinal tract motility malabsorption, loss of appetite and enlarged tongue (Green Span and Dong, 2004 and Rajab et al., 2015).
Salivary glands; parotid, submandibular and sublingual secret saliva, which acts not only as the first biological media for food but also the first line of defense for oral cavity soft tissues and teeth (Cecchini et al., 2009 and El-Bassouny, 2012). Many studies have correlated between salivary gland dysfunction and autoimmune thyroiditis (Olver, 2006). Hayat et al. (2010) noticed that most of hypothyroid patients were presented by salivary glands enlargement. They considered this enlargement as a useful clue to the diagnosis of hypothyroidism. Most of the available literatures were concentrated on the physiological and biochemical changes of salivary glands secondary to hypothyroidism. In contrast, their histological changes have not been sufficiently studied. So, the aim of this work is to study the possible histological alterations that may occur in parotid gland structure in hypothyroid adult male albino rats and the role of thyroid hormone supplementation.

Materials and Methods

Animal and design
Thirty adult healthy male albino rats (3-5 months) weighing 200-250 gm were used in this study. All animals were kept in clean, properly ventilated cages under similar environmental conditions. The animals were divided equally into three groups: Group I (control), Group II (treated) and Group III (thyroid hormone supplemented group). Animals of hypothyroid group were received carbimazole (NeoMercazol); obtained from Chemical Industries Development; antithyroid drug at a dose of 0.05 mg/kg body weight/day for successive 3 weeks, the drug was given by gastric tube (Dakine et al., 2000). Animals of thyroid hormone supplemented group were received carbimazole (NeoMercazol) antithyroid drug at a dose of 0.05 mg/kg body weight/day for successive 3 weeks then treated with thyroxin in the form of Levothyroxine sodium (10µg/100gm/BW) for another successive 3 weeks. Thyroid hormone was obtained from Glaxo Wellcome Company. At the time of sacrifice, the animals were anaesthetized by ether inhalation. Blood samples from rats’ orbital veins of all groups were collected for evaluation of serum total T3, total T4 and thyroid stimulating hormone (TSH). Intracardiac perfusion was done by of 1.5% glutaraldehyde buffered with 0.1 M Sodium cacodylate at pH 7.4 for partial fixation of parotid. With extended head. Right and left parotid glands of rats in all groups were dissected out carefully and processed for light, electron microscopic examination and morphometric study.

Light microscopic study

Hematoxylin and Eosin Stain and Mallory’s trichrome
Specimens were fixed in formol saline and were processed for paraffin sections of 5μm-thick stained with Haematoxylin and Eosin (H & E) and Mallory’s trichrome (Bancroft and Gamble, 2008).

Immunohistochemical Study (Bcl-2)
Immunohistochemical reactions were carried out according to (Kiernan, 2000) using the avidin biotin peroxidase system for localization of Bcl-2 (antiapoptotic marker). Paraffin sections were cut and deparaffinized in xylene for 30 minutes and re-hydrated. The endogenous peroxidase activity was blocked, rinsed in distilled water and then washed by phosphate buffered saline (PBS). The PH should be 7.2. Slides were then dried, covered by protein blocking reagent (normal rabbit serum) followed by covering with primary antibody, then the section was covered with biontynlated secondary anti-mouse antibody washed in PBS. Avidin biotin reagent (ABR) was prepared, slides were dried then were taken to distilled water, counterstaining with Mayer’s Haematoxylin, washed, dehydrated, left to dry, mounted, covered with glass covers and then examined.
Results:
The positive results were indicated by brown colouration.

Transmission electron microscopic study
Ultra-thin sections (70-80 μm) were cut and mounted on copper grids. The grids were double stained with uranyl acetate and lead citrate for examination with transmission electron microscope (Joel TEM) at Histology and Cell Biology Department, Faculty of Medicine, Zagazig University according to Woods and Stirling (2002).

Image Analysis and Morphometric Study
H&E and Mallory trichrome stained sections were morphometrically analyzed using Leica Qwin 500 Image Analyzer Computer System (England), at Pathology Department, Faculty of Dentist, Cairo University. Ten high power fields were taken for each section of all groups.

Biochemical (Hormonal) Analysis:
At the time of sacrifice, blood from each animal was rapidly collected from orbital vein transferred to centrifuge tubes without anticoagulant, and serum was separated by low-speed centrifugation (1500 xg 5 min). The serum samples were stored at -20°C until the analysis time. Serum thyroid hormones [total T3, total T4 and thyroid stimulating hormone (TSH)] levels were measured by in vitro diagnostic radioimmunoassay with the immunolite 2000 analyzer for the quantitative measurement (El-Wakf et al., 2009). (Mean serum TT3 ranged from 38.96-41.04 μg/dl, mean serum TT4 ranged from 3.1-5.3 μg/dl and mean serum TSH ranged from 0.114-0.126 μU/ml.

Statistical Analysis:
The data obtained (serum total T3, serum total T4, serum TSH) and area percent of collagen for all groups were expressed as means (X̄) and standard deviations (SD) and subjected to statistical analysis using one-way analysis of variance (ANOVA) for comparison between the different groups (more than two groups). Followed by least significant difference test (LSD), for comparison between different groups, i.e. to find the statistical difference between groups when ANOVA was statistically significant (P value <0.05 was considered statistically significant) (Kostogrys et al., 2006).
All statistical analysis were done using the Statistical Package for the Social Sciences (SPSS) version 19 packages.

Results

Light and electron microscopic results

Group I (Control Group): Examination of H&E stained sections from control animals showed that the parenchyma was formed of numerous closely packed serous acini with narrow interstitium in between. Each serous acinus consists of a single layer of pyramidal cells with basal rounded nuclei and homogenous acidophilic cytoplasm, surrounding a lumen. Among the acini, Striated ducts were present and lined by a single layer of columnar epithelium with mostly round nucleus (Fig. 1). Mallory’s trichrome stained sections of control group showed thin aggregation of collagen fibers inbetween the acini of parotid gland (Fig. 2). Immunohistochemical stained sections of control group showed strong immune reaction for bcl2 in the cytoplasm of parotid cells (Fig. 3). Examination of the ultrathin sections showed that serous acinar cells of the control group of parotid gland had ovoid euchromatic nuclei with peripheral thin rim of heterochromatine and prominent nucleoli. Their apical cytoplasm contained many membrane bounded electron lucent secretory granules. Few electron dense secretory granules were also seen (Fig. 4).
**Fig. 1:** H&E stained section of the control group showing the normal serous acini (a) and striated duct (d). The acini are separated by thin interacinar connective tissue (arrow heads). Striated ducts appear lined by a single layer of columnar epithelium with mostly round nucleus (arrows). (X 200)

**Fig. 2:** Mallory Trichrome stained sections of control group (group I) showing blue stained collagen fibers (arrow heads) in between lobules, around blood vessels and interlobular ducts (arrows) (X 200).
Fig. 3: Immunohistochemical stained sections of control group showing strong immune reaction for bcl2 in the cytoplasm of parotid cells (arrows) (X 200)

Fig. 4: Ultrastructure photomicrograph showing control acinar cell with euochromatic nucleus (N), electron dense (d) and electron lucent (L) secretory granules (Figure X 15000).
Group II (hypothyroid Group): Histological examination of the parotid of H&E stained sections parenchyma contained many serous acini. Most of them had irregular outlines and were widely separated. Some acinar cells had deeply stained nuclei and vacuolated cytoplasm. Congested blood vessels and cellular infiltration were seen in the interstitial space. The interlobular ducts appeared dilated (Fig. 5). Mallory’s trichrome stained sections clarified the presence of extensive collagen fibers in parotid gland of group II (Fig. 6). Immunohistochemical stained sections of hypothyroid group (Fig. 7) showed weak immune reaction for bcl2 in the cytoplasm of parotid cells Ultrathin sections of hypothyroid group showed that some acinar cells had irregular heterochromatic nuclei. Their cytoplasm contained markedly dilated rough endoplasmic reticulum (RERs) (Fig. 8). Some acinar cells showed variable sized vacuoles in their cytoplasm. (Fig. 9). Other cells showed apoptotic nuclei (n) (Fig. 10)

(Fig. 5). H&E stained section of hypothyroid group (group II) showing that most of the acini have irregular outlines (arrows), contain darkly stained nuclei (arrow heads) and vacuoles (v). Dilated interlobular ducts (d), cellular infiltration (i) and congested blood vessels (BV) are seen. (X 200)
Fig. 6: Mallory Trichrome stained sections of hypothyroid group (group II) showing collagen fibers in between lobules (arrow heads), around blood vessels and interlobular ducts (arrows) (X200)

Fig. 7: Immunohistochemical stained sections of hypothyroid group showing weak immune reaction for bcl2 in the cytoplasm of parotid cells (X 200)
Fig. 8: Ultrastructure photomicrograph showing acinar cells of group II contain irregular heterochromatic nucleus (N) and show multiple dilated rough endoplasmic reticulum (r) (X 4000)

Fig. 9: Ultrastructure photomicrograph showing acinar cells of group II contain multiple vacuoles (v). (X 4000)
Fig. 10: Ultrastructure photomicrograph showing acinar cells of group II showing multiple dilated rough endoplasmic reticulum (r). Some cells have normal nuclei (N). Other cells appear with apoptotic nuclei (n) (X 4000)

Group III (thyroid hormone supplemented): Histological examination of H&E stained sections of parotid gland of thyroid hormone supplemented group showed that most of acini and ducts were apparently normal with some acini had irregular outline. Some acinar cells still contained vaculated cytoplasm (Fig. 11). Collagen fibers were apparently decreased in group III in comparison to group II (Fig. 12). Collagen fibers density was confirmed by morphometrical results (table 1). Immunohistochemical stained sections of thyroid hormone supplemented group revealed moderate reaction in the cytoplasm of parotid cells (Fig. 13). Ultrathin sections of thyroid hormone supplemented glands showed that some acinar cells were apparently normal, while others still had some dilated rough endoplasmic reticulum (RERs) and some vacuoles in their cytoplasm (Fig. 14, 15).
**Fig. 11:** H&E stained section of thyroid hormone supplemented group showing apparent normal acini (a) and ducts (d). (X 200)

**Fig. 12:** Mallory Trichrome stained section of thyroid hormone supplemented group (group III) showing collagen fibers (arrow heads) in between lobules, around blood vessels and interlobular ducts (arrows). (X 200)
Fig. 13: Immunohistochemical stained sections of thyroid hormone supplemented group showing moderate reaction in the cytoplasm of parotid cells. (X 200)

Fig. 14: Ultrastructure photomicrograph of thyroid hormone supplemented group (group) showing acinar cells with few dilated rough endoplasmic reticulum (r) and few vacuoles (v). (X 3000)
Fig. 15: Ultrastructure photomicrograph of thyroid hormone supplemented group (group) showing acinar cells with few dilated rough endoplasmic reticulum (r) and few vacuoles (v). (X 3000).

**Morphometric and statistical results**

Highly statistically significant increase in the mean area percent of collagen fibers of the parotid gland was detected in the hypothyroid group as compared to the control group and thyroid hormone supplemented group. No statistically significant difference between thyroid hormone supplemented group and the control group (Table 1). Highly statistically significant decrease in the mean values of total T3 and total T4 and highly significant increase in the mean values of TSH was detected in the hypothyroid group as compared to the control group. Highly statistically significant increase in the mean values of total T3 and total T4 and highly significant decrease in the mean values of TSH in thyroid hormone supplemented group (p<0.001) when compared to those of hypothyroid group. However, there was no statistical significant difference between thyroid supplemented group and control group (p>0.05) (Table 2).
Table (1): Comparisons between mean values of percent of collagen area in the different studied groups using ANOVA (analysis of variance) test.

<table>
<thead>
<tr>
<th></th>
<th>Mean ± SD</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.06±3.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypothyroid group</td>
<td>22.76±5.67</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thyroid hormone supplemented group</td>
<td>8.90±4.08</td>
<td>37.51</td>
<td>&lt;0.001**</td>
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</table>

LSD (least significance difference for comparison between groups)

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Hypothyroid group</th>
<th>Thyroid hormone supplemented group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypothyroid group</td>
<td>&lt;0.001**</td>
<td></td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>Thyroid hormone supplemented group</td>
<td>0.63 (NS)</td>
<td>&lt;0.001**</td>
<td></td>
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</tbody>
</table>

*Significant (p<0.05)  **Highly Significant (p<0.001)

Table (2): Comparisons between mean values of serum thyroid hormones levels (total T3, T4 and TSH) in the different studied groups using ANOVA (analysis of variance) test.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Parameter</th>
<th>Control Mean±SD</th>
<th>Hypothyroid group Mean±SD</th>
<th>Thyroid hormone supplemented group Mean±SD</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total T3 (µg/dl)</td>
<td>39.2±0.52</td>
<td>24.45±1.15</td>
<td>34.2±0.56</td>
<td>814.57</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>Total T4 (µg/dl)</td>
<td>4.11±0.56</td>
<td>2.53±0.15</td>
<td>3.77±0.51</td>
<td>27.83</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>TSH (µIU/ml)</td>
<td>0.13±0.003</td>
<td>0.24±0.02</td>
<td>0.15±0.032</td>
<td>273.08</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>

LSD (least significance difference for comparison between groups)

<table>
<thead>
<tr>
<th>Group</th>
<th>Parameter</th>
<th>Control -</th>
<th>Hypothyroid group</th>
<th>Thyroid hormone supplemented group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total T3 (µg/dl)</td>
<td>&lt;0.01af7 a</td>
<td></td>
<td>0.116. a (NS)</td>
</tr>
<tr>
<td></td>
<td>Total T4 (µg/dl)</td>
<td>&lt;0.001**a</td>
<td></td>
<td>0.37 a (NS)</td>
</tr>
<tr>
<td></td>
<td>TSH (µIU/ml)</td>
<td>&lt;0.001**a</td>
<td></td>
<td>0.12 a (NS)</td>
</tr>
<tr>
<td></td>
<td>% of collagen area</td>
<td>&lt;0.001**a</td>
<td></td>
<td>0.63 a (NS)</td>
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</table>

*: significant (p<0.05)  **: highly significant (p<0.01). NS: non-significant.

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DISCUSSION

Previous studies proved that thyroid gland rendered hypo-functional when treated with MMI (Hayat et al., 2010). The functional state of thyroid gland was established by histological changes and serum level of T3, T4 and TSH hormones; it was postulated that the drug acts as a false substrate for thyroid peroxidase, thus blocking the iodination of tyrosine residues within thyroglobulin (Čakić-Milošević et al., 2004).

In our experimental model, development of hypothyroidism was confirmed both by histological changes in the gland and T3, T4 and TSH serum levels. Significant decrease in T3, T4 and increase in TSH serum levels was indicative that the quantity and duration of treatment was sufficient to induce hypothyroid status in the experimental group of rats.

Examination of the hypothyroid group (group II) showed that most of the serous acini had irregular outlines and were widely separated. Some acinar cells contained darkly stained nuclei and cytoplasmic vacuoles. Cellular infiltration and many collagen fibers around congested blood vessels were also noticed.

These histological changes in the glands may be due to the adverse effects of hypothyroidism upon metabolic systems within the cell. Serous cells of the parotid gland are specifically affected by hypothyroidism. Our results could imply the existence of functional relationship between salivary and thyroid glands.

Rodriguez et al. (2009) stated that the salivary glands have a high metabolism and they require large blood flow to produce saliva and in a hypothyroidism condition, the energy supply commits the composition, synthesis, and secretion of saliva. Furthermore, studies show that thyroid hormones have influence on tissues, because they facilitate DNA transcription resulting in new protein synthesis. It means that in a hypothyroidism, significant changes occur in the structure of salivary glands (Jesus et al., 2015).

Microscopic findings of the hypothyroid rat glands showed atrophy of acini and partial replacement of the parenchyma with a connective tissue component. The same results were obtained by Oncu et al. (2004) with sublingual gland of thyroidectomized rats and Hayat et al. (2010) with parotid gland of hypothyroid rats induced by methimazole. Hayat et al. (2010) found an increase in the interacinar and intralobular connective tissue mass together with small and more irregular serous acini.

In the present study, the nuclei of parotid gland cells were darkly stained in the experimental animals. Ashour (1998) reported that the amount of euchromatin was used as an indicator of the metabolic activity of cells; conversely a high proportion of heterochromatin indicates a cell with low metabolic activity.

Examination of ultrathin sections of hypothyroid group showed acinar cells with irregular heterochromatic nuclei, dilated rough endoplasmic reticulum, degenerated mitochondria and cytoplasmic vacuoles. These structural changes were consistent with Yang et al. (2015) who found similar changes in hippocampus of hypothyroid rats. Abo-Elghait et al. (2011) explained these results by the direct effect of thyroid hormone at the transcription level by binding to nuclear receptors leading to inhibition of incorporation of labeled amino acids into proteins and decrease in RNA content in the nervous tissue of hypothyroid young animals.
Also, Koromilas et al. (2010) reported that thyroid hormones have been played an important role in the regulation of mitochondrial function in several tissues. Furthermore, mitochondria seem to participate in hypothyroid-induced apoptotic phenomena that take place in hippocampal neurons of developing rats. In addition, Bhanja and Chainy (2010) reported that propylethiouracil (PTU) induced hypothyroidism was able to induce oxidative stress in rat cerebellum that resulting in tissue damage and apoptosis.

In the current work, Mallory’s trichrome stained sections clarified the presence of extensive collagen fibers in parotid gland of group II. While, Collagen fibers were apparently decreased in group III in comparison to group II. Fibrosis of the gland was confirmed by morphometrical results. Khalawi et al. (2013) explain the noticed signs of inflammation as cellular infiltrations and fibrosis by the ability of oxidative stress to stimulate the expression of genes involved in collagen biosynthesis. Also, Anan et al. (2012) reported that oxidative stress were responsible for increasing evidence for the immunologic role and the activation of macrophages to become high secretory cells and release several factors, such as: interleukin-1, tumor necrosis factor, prostaglandins, ROS, lipid peroxides. These metabolites were injurious to other cells and their release was chemotactic for other cells as neutrophils and lymphocytes.

The same results were obtained by Abou-Elghaita et al. (2011) who stated that thyroid deprivation cause of changes in the levels of lipid peroxidation and antioxidant enzyme activities which were determined in different tissues of hypothyroid rats, causing the functional disorder of these tissues.

In the current work, immunohistochemical examination for bcl2 was performed. In the control group, immunohistochemical stained sections showed strong immune reaction for bcl2 in the cytoplasm of parotid cells. Concerning hypothyroid group; weak immune reaction for bcl2 in the cytoplasm of parotid cells was detected. While, immunohistochemical examination for bcl2 of thyroid hormone supplemented group revealed moderate reaction in the cytoplasm of parotid cells. BCL-2 family proteins are the regulators of apoptosis (antiapoptotic factors), but also have other functions. BCL-2 proteins have critical roles in normal cell physiology related to neuronal activity, autophagy, calcium handling, mitochondrial dynamics and energetics, and other processes of normal healthy cells. BCL-2 is localized to the outer membrane of mitochondria, where it plays an important role in promoting cellular survival and inhibiting the actions of pro-apoptotic proteins. (Hardwick and Soane, 2013)

In this study, supplementation of thyroid hormone showed amelioration of most degenerative changes that occurred in the parotid gland.

CONCLUSIONS:

Hypothyroidism produces histological alterations in glandular tissue of parotid gland indicating that thyroid hormones are essential for its normal function. The results of our experiment support the idea expressed by other investigators that the thyroid-salivary gland relationship exists and is mediated through thyroid hormones.
RECOMMENDATIONS

Early diagnosis of hypothyroidism with rapid interference.

Conflict of interest

The authors declare that they have no conflict of interest.

REFERENCES


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