Original Article

Alpha Lipoic Acid Exerts a Therapeutic Effect on Parotid Tissue in a Hypothyroidism Rat Model by LC-3 Suppressing Autophagic Cell Death (Immunohistochemical, Morphometric, Ultra-structural and Biochemical Study)

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ABSTRACT

Introduction: Hypothyroidism is a common thyroid problem that can be experimentally induced by the anti-thyroid drugs as Carbimazole. Hypothyroidism is recorded to affect the salivary glands.

Aim of the Work: To assess the therapeutic ability of Alpha Lipoic Acid (ALA) on the parotid tissue in a hypothyroidism experimentally induced rat model.

Material and Methods: The animals were distributed into: (1) Control group was subdivided into: group 1: got an oral dose of distilled water daily for 3-weeks, group 2: received 2 ml/kg/day corn oil dosage for 4-weeks, (2) Hypothyroidism-Induced Group received an oral dose of 1.35 mg/kg/day Carbimazole for 3-weeks, (3) Hypothyroidism-ALA group: received an oral dose of 1.35mg/kg/day Carbimazole for 3 weeks then a 60 mg/kg/day ALA oral dosage for another 4-weeks, (4) ALA Group: received a dose of 60mg/kg/day ALA orally for 4-weeks. Estimation of the body, gland weights, T3, T4 and TSH hormones levels were performed. A histopathological and statistical study of the parotid tissue was performed.

Results: In hypothyroid rats, the acinar cells showed irregular heterochromatic nucleus, cytoplasmic vacuolations and cellular infiltration. The striated and interlobar ducts showed dilatation and hyperplasia. In comparison with the control group, the hypothyroid rats exhibited a significant rise in area percentage of LC3 immune-expression, diameters of acini and ducts and TSH level with a significant decrease in T3 level, the numbers of the ducts and acini. On ALA use, all those findings were improved.

Conclusion: ALA serves as a good therapeutic agent leading to an obvious improvement in the parotid gland histopathological features.

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Key Words: Autophagy, hypothyroidism, parotid gland, ultra-structure.

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INTRODUCTION

Hypothyroidism is the most common thyroid condition. It is correlated to various metabolic abnormalities. Hypothyroidism can occur due to deficient iodine consumption, thyroid gland injuries, autoimmune syndromes, and a disturbed action of pituitary gland[1]. Hypothyroidism can be induced by the anti-thyroid drug Carbimazole used in the treatment of human hyperthyroidism[2].

Carbimazole is one of several thionamide drugs used for the treatment of different diseases causing hyperthyroidism in adults and children. Carbimazole is converted into the active form Methimazole (MMI) after its ingestion[3]. The adverse reactions of Carbimazole include allergy, joint pain, swelling, taste or smell abnormality or gastrointestinal disturbances[4].

Many studies linked between hypothyroidism and parotid gland affection. It is indicated that most of the hypothyroid patients present with salivary glands enlargement[5]. Thyroid function and oral health are closely associated. Also, a correlation exists between salivary gland dysfunction and autoimmune thyroiditis[6].

The medical uses of natural herbs are gradually increasing due to their antioxidant, anti-inflammatory, anti-bacterial and anti-cancer capabilities[7]. ALA is demonstrated to decrease oxidative stress-caused cells' injury in numerous in-*vitro* and in-*vivo* models[8,9].

In fact, ALA is a critical co-factor for numerous enzymes facilitating chief metabolic responses for example; acetyl-CoA formation, fatty acid metabolism, tri-carboxylic acid cycle (α-keto glutaric acid to succinyl-CoA conversion), and nucleotide formation[10]. It also plays a role as an antioxidant through its capability to hunt free radicals and tolerate the intrinsic cells' antioxidant security system. A protective role of ALA on radiation-induced thyroid gland[11], salivary gland[9], and intestinal injury[12] is reported.

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Accordingly, this research aimed at declaring and evaluating the possible curative role of ALA when dealing with parotid gland affection due to hypothyroidism.

MATERIAL AND METHODS

Test Chemicals

- **Carbimazole:** SML0931 (Sigma -Aldrich, St Louis Co., MO, USA) 5mg tablets.
- **ALA:** (Thiotacid / Thiocticacid) 300 mg capsules.

They were obtained from Eva-Pharm Pharmatheutical Company - Egypt.

Experimental Animals

Thirty-six adult male albino rats (6 weeks old, 180–200 g body weight) were acclimatized for 2 weeks and then haphazardly distributed into four groups (n = 9).

The animals were kept in the animal house, faculty of medicine, Zagazig University, with availability of food and water ad libitum. The housing settings were kept at a stable temperature (24 ± 1 °C), humidity (55 ± 5 %), ventilation rate (18 times/h), and a 12-h light/dark cycle. The rats were kept in plastic cages (four per cage) with soft chip sheet. The cage size of 47× 30× 15cm, was suitable for 4 animals' growth. During the study, the wood chips were changed every 3 days. The healthiness state of the rats was observed every day. The procedures to decrease pain or discomfort were carried out. The animals' study was accepted by the Institutional Animal Care and Use Committee of Zagazig University (ZU-IACUC/3/F/118/2020), and all the study procedures were in accordance with the strategies stated in the Guide for the Care and Use of Laboratory Animals.

Experimental Design

Thirty-six animals were equally assigned to the following groups:

Control: where the rats were distributed to 2 subdivisions:

- The first one: contained 4 animals that were given an everyday oral dose of distilled water (Carbimazole's vehicle) for 3 weeks.
- The second one: contained 5 animals that were given an everyday oral dose of 2 ml/kg, corn oil (ALA vehicle) for 4 weeks[13].

Hypothyroidism- induced: in which the rats received 1.35 mg/kg/day dose Carbimazole orally for 3 weeks for hypothyroidism induction[14].

Hypothyroidism- induced + ALA: where the animals received a daily oral dose of 1.35mg/kg Carbimazole for 3 weeks followed by an oral dose of 60 mg/kg/day ALA for the successive 4 weeks.

ALA: where the animals received a dose of 60mg/kg/day ALA orally for 4 weeks[15].

- All the experimental animals received their oral doses via intragastric intubation.

Considering that all animals in all groups were having the same weight nearly, and they were randomly distributed between the experimental groups, we compared the rats' body weight by the end of the experiment. So, by the experiment termination, all animals were weighed using a sensitive scale then an intra-peritoneal injection of 120 mg/ kg Sodium Thiopental was used[16]. Then, samples of blood were directly aspirated by cardiac puncture[17] and left till spontaneous coagulation. The parotid tissue was directly dissected and weighed using a sensitive scale. The glands were then fixed in 10% formalin for light microscopic examination or with 4% cold Glutaraldehyde (at 4OC) in a buffered cacodylate solution (pH 7.4) for electron microscopic examination.

Biochemical Analysis

Blood samples were directly aspirated by cardiac puncture[17] and left till spontaneously coagulated. From each animal, a sample of 2 ml blood was obtained into a tube for thyroid hormones assays. The blood specimens were kept for serum separation, then centrifuged at 3000 r. p. m for 15 minutes. The serum specimens were reserved at -200C[18] for hormonal analysis by the estimation of serum T4 and T3 according to the guidelines[19]. The hormonal kits were achieved from Calbiotech INC (CBI), USA. The data were collected, listed and analyzed following standardized statistical methods.

Histological Techniques

Light microscopic study was done for

a) Observation of the general histopathological characters on Hematoxylin and Eosin (H&E) staining:

All steps were conducted at Pathology Department, Faculty of Medicine, Zagazig University. Consistent with usual measures[20], fixed parotid samples in 10% neutral buffered Formalin and in Paraffin were prepared. Sectors of 5µm thickness were mounted on glass slides, deparaffinized in Xylene and stained by Hematoxylin and Eosin stain (H&E).

The stained sections were examined and analyzed by light microscopy (LEICA ICC50 W) in Image Analysis Unit of Human Anatomy and Embryology Department, Faculty of Medicine, Zagazig University. Leica Q Win plus Image Analysis System (Leica Micros Imaging Solutions Ltd, Cambridge, UK) was used for the morphometrical studies.

b) Immunohistochemical study (IHC) by Light Chain-3 (LC3) autophagy marker:

The prepared paraffin-fixed parotid gland specimens (5 mm in thickness) were heated at 67oC for 45 minutes. The sections were treated by an antigen-retrieval method consisting of water bath heating at 90oC for ten min. and cooling for five min. for antigens' recovery, and then

rehydrated in a settled sequence of alcohol. The samples were preserved with 3% H2O2 for 15 min. at 37oC to stop the endogenous peroxidase action. Broad binding was stopped with 5% bovine serum albumin at room temperature for 20 min. Sample incubation with antibodies contrary to the microtubule-associated protein 1 light chain 3 (LC3) (1:100) was done over-night. The parotid gland specimens were cleaned with Phosphate Buffered Saline (PBS), then incubated with the secondary antibody (1:500 in PBS) directly for 30 min. Lastly, the Diaminobenzidine kit was used to visualized the antibody binding where the parotid gland samples were examined by light microscope. ImageJ software was used to estimate the proportion of the area stained with brown color to the whole area[21].

c) Transmission electron microscopic study (TEM):

The samples (1 mm3 thick) of parotid gland were preserved in a mix of 2.5% Glutaraldehyde and 2.5% Paraformaldehyde, in Phosphate buffer for 24 hours, post-fixed in 1% Osmium Tetra-Oxide, dehydrated and preserved in Resin. The samples were cut, then sectioned into semi-thin and ultrathin sections. The semi-thin sections (1µm) were stained by toluidine blue (TB) then inspected under a light microscope. The ultra-thin sections were transferred to copper grids for staining with Lead Citrate and Uranyl Acetate. The prepared samples were examined by a transmission electron microscope (TEM) at the Electron Microscopy Unit, Mansoura University using JEM-2100 transmission electron microscope to detect the ultra-structural changes.

Morphometric Analysis

Ten non-overlapping fields from different sections of each animal in each group were used to measure:

The mean numbers of acini and ducts in sections-stained with Hx&E (x100).

The mean diameters of acini and ducts in sections-stained with Hx&E (x100).

This was done by using Leica ICC50 W-software image analysis computer system (Cambridge, England) at Human Anatomy and Embryology Department, Faculty of Medicine, Zagazig University.

Statistical Evaluation

The obtained data were charted, statistically analyzed and represented graphically. The values were presented as mean and standard deviation (SD). Multiple comparisons of the groups were done by using one-way analysis of variance ANOVA and Tukey's post hoc tests. The significance level was at $p \leq 0.05$. The Statistical analysis was carried out by statistical analysis system SAS (Cary, NC, USA).

RESULTS

Results of Biochemical Assays

Regarding T3 hormone level, a significant reduction ($p<0.05$) was detected in hypothyroidism-induced group in contrast with the control and ALA groups. A non-significant reduction in T4 hormonal level was observed on comparing hypothyroidism-induced group with the other groups. Regarding TSH level, a significant increase ($p<0.05$) was detected in hypothyroidism-induced group in comparison with the control and ALA groups. Meanwhile hypothyroidism-induced + ALA group did not vary significantly ($p>0.05$) on comparison with the control and ALA groups (Table 1).

Body, Parotid gland and Relative Weights

By the end of the study, Hypothyroidism-induced group present a statistically significant drop in the body weight in comparison with the control, ALA and hypothyroidism-induced+ ALA groups. Both right and left glands' weights are significantly reduced in hypothyroidism-induced and hypothyroidism- induced +ALA groups on comparing with the control and ALA ones. A statistically significant difference was noticed on comparing hypothyroidism-induced and hypothyroidism-induced +ALA groups. Regarding the relative weight (the ratio between the parotid gland weight and the body weight), a significant decrease was observed on comparing hypothyroid -induced and hypothyroid-induced +ALA groups with the control and ALA groups (Table 2).

No significant differences were observed regarding the biochemical assays and body, parotid and relative weights on comparing the control and ALA groups. So, both groups were considered control in discussing the histopathological results.

Histopathological Results

a) H and E Sections

The control group showed normal parotid tissues. The parotid gland was formed of lobules; each lobule contained regular densely packed serous acini and striated ducts with a fine network of interlobular connective tissue. Each serous acinus was lined by one row of long pyramidal epithelial cells surrounding a central lumen. Slim fibrous connective tissue septa were present among the acini. A single layer of short columnar epithelial cells lined the striated ducts existing among the acini (Figure 1a). In hypothyroidism-induced group, the acinar cells lost their normal arrangement. The cytoplasm showed moderately large several vacuoles in most of acinar cells and cellular infiltration. The striated and interlobar ducts showed dilatation, became lined with more than one layer of cells (hyperplasia) with periductal blood vessels exhibited massive congestion and surrounding massive fibrosis (Figure 1b). In the hypothyroidism-induced + ALA group, the glandular tissue reestablishes the majority of the typical acinar architecture and the vacuoles in the acinar epithelial cells were decreased. The ducts restored their normal epithelial lining and the fibrosis around the ducts and blood vessels diminishes (Figure 1c). In ALA-group, the glandular tissue arrangement and structure were nearly the same of the control group (Figure 1d).

b) The impact of Alpha-lipoic Acid treatment on the immune-expression of autophagy marker (LC3)

The immune-histochemical study of autophagic proteins in control parotid gland exhibited regular ducts and acini, with a negative LC3 immune-expression (Figure 2a). Markedly, raised LC3 levels in Carbimazole induced hypothyroidism group were settled by a significant increase in the area percent of brown staining, indicating a stronger LC3 immune-expression (Figure 2b) compared with the control group (P <0.005). On the other hand, the use of ALA revealed a decrease of LC3 expression, settled by a significant reduction in area percent of LC3 (Figure 2c) immune-expression in comparison with hypothyroidism-induced group. In ALA- group, the glandular tissue exhibited regular ducts and acini, with a negative LC3 immune-expression (Figure 2d).

c) Semi-thin Toluidine Blue stained sections

In the control group, pyramidal acinar cells rested on a basement membrane. The nuclei were normal with prominent nucleoli. However, some cells were binucleated. The cytoplasm showed numerous secretory granules. The striated ducts were normal and lined by a single layer of cells. The blood vessels were also normal (Figure 3a). In hypothyroidism-induced group, the acinar cells showed deeply stained nuclei. The striated ducts showed hyperplasia. Congested blood vessels were also noticed (Figure 3b). In hypothyroidism-induced + ALA group most of acinar cells regained their normal pyramidal shape and normal nucleus with prominent nucleolus, few cells still had darkly stained nuclei. The cytoplasm became filled with secretory granules. The striated ducts appeared normal and were layered by a single row of cells but were still surrounded by a minimal fibrosis. The blood vessels were minimally congested (Figure 3c). In ALA- group, there was no obvious changes in the glandular tissue arrangement and structure other than that was observed in control group (Figure 3d).

d) Ultra-structural Results

Ultra-thin sections in the adult rats' glandular tissue of the control group exhibited acinar cells with euchromatic rounded nuclei, a steady nuclear membrane and a peripheral thin rim of heterochromatin. The rough endoplasmic reticulum (RER) and mitochondria were normal. Intracellular electron dense and undeveloped electro-lucent secretory granules were present. The quantity of the highly electron dense granules was more than that of the immature electro-lucent ones. The intercellular junctional areas showed a limited intercellular canaliculus (Figures 4,5a). The ultra-thin sections of hypothyroidism- induced group showed that the acinar cells had an irregular nuclear and cell membrane. The cytoplasm had highly electron dense, discreetly electron dense, and electro-lucent secretory granules. The rough endoplasmic reticulum became dilated and disarranged. Small electron lucent. The intercellular junctional areas showed wide intercellular canaliculi (Figures 4,5b). The ultra-thin sections of hypothyroidism-induced + ALA group parotid gland showed that the acinar cells restore their regular rounded nuclei with a peripheral thin rim of heterochromatin. The cytoplasm contained highly electron dense, relatively electron dense and electro-lucent secretory granules. The cytoplasmic granules were few. Some cells' cytoplasm was nearly empty of any secretory granules. Most of the RER became non-dilated, highly developed while few show dilatations. The intercellular junctional areas showed that the intercellular canaliculi were still widened. (Figures 4,5c). In ALA- group, there was no obvious changes in the glandular tissue arrangement and structure other than that was observed in control group (Figures 4,5d).

Morphometric Results

Interestingly, significant increases were observed in the diameters of acini and ducts on comparing the Hypothyroidism-induced and Hypothyroidism- induced + ALA groups with the control and ALA groups. Furthermore, significant differences (p<0.05) were noticed between Hypothyroidism- induced and Hypothyroidism - induced + ALA groups. There was a significant decrease in the number of acini and ducts on comparing the Hypothyroidism-induced and Hypothyroidism- induced + ALA groups with the control and ALA groups. Also, significant differences (p<0.05) were detected between Hypothyroidism- induced group and Hypothyroidism-induced + ALA group. On the other hand, there was a significant rise in area percentage of LC3 positive immune reaction in hypothyroidism-induced group in comparison with control and ALA groups. A significant decrease in the area percentage of LC3 immune reaction was noticed on comparing the hypothyroid-induced+ ALA group with hypothyroidism- induced group (Table 3).

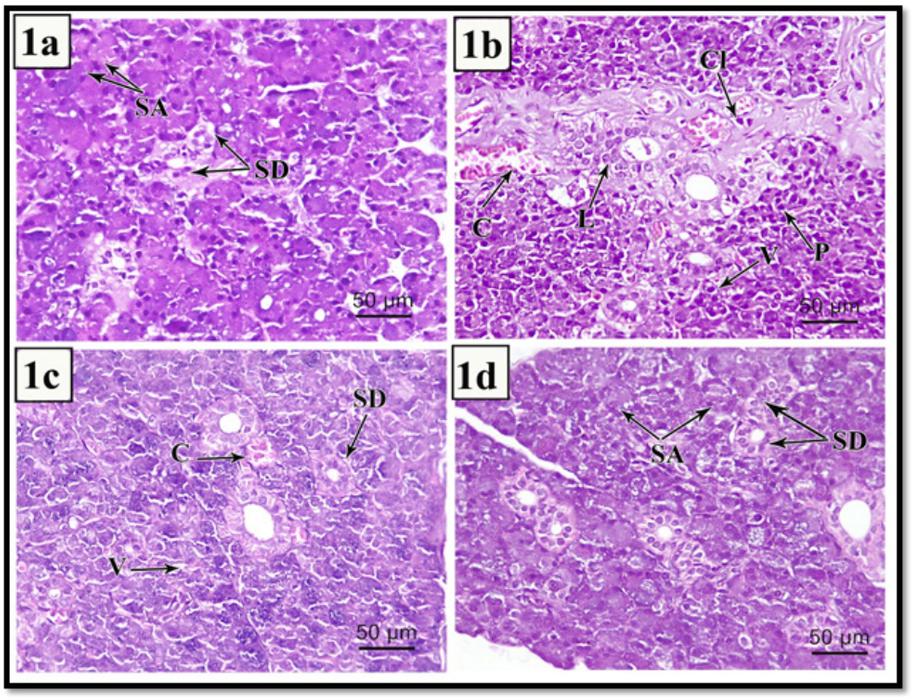

Fig. 1: Illustrative photomicrographs of H&E-stained sections in adult rats' parotid glands from the different experimental groups. (1a) A Photomicrograph of control group showing: normal serous acini (SA). Among the acini, striated ducts are observed (SD) lined by a single layer of columnar epithelium. (1b) A photomicrograph of hypothyroidism-induced group showing: a periductal congestion (C). The striated ducts are lined by more than one layer of cells (L). Shrunken acinar cells (P) with cellular infiltration (CI) and vacuolations (V) are observed. (1c) A photomicrograph of hypothyroidism – induced + ALA group showing: few epithelial cytoplasmic vacuolations (V), a little periductal congestion (C) and the striated ducts exhibit normal shape (SD). (1d) A Photomicrograph of ALA- group showing: normal serous acini (SA). Among the acini, striated ducts are observed (SD) lined by a single layer of columnar epithelium. (Hx & E x 400, Scale bar 50 µm).

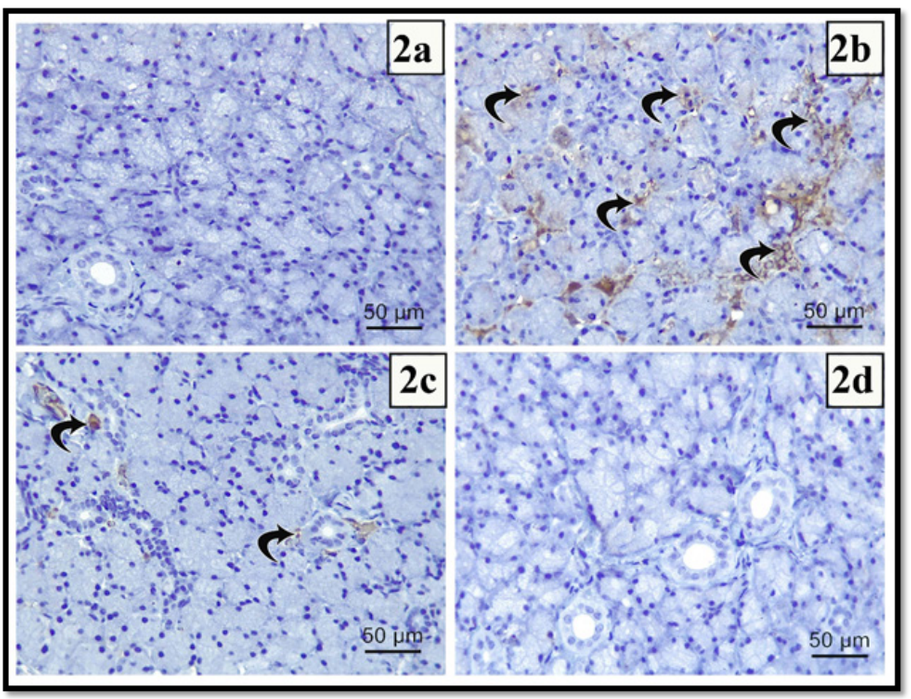

Fig. 2: Photomicrographs show the immune-expression of autophagy-associated proteins LC3 in rats' parotid glands of the experimental groups. A positive immune-expression is specified by brown discoloration. (2a) The Control group shows normal ducts and acini with a negative LC3 immune-expression. (2b) Elevated levels of LC3 are detected in the hypothyroidism - induced group. (2c) The ALA use with hypothyroidism-induced group reveals a downregulation of LC3. (2d) The ALA- group shows normal ducts and acini with a negative LC3 immune-expression (Light Chain-3x 400, Scale bar 50 µm)

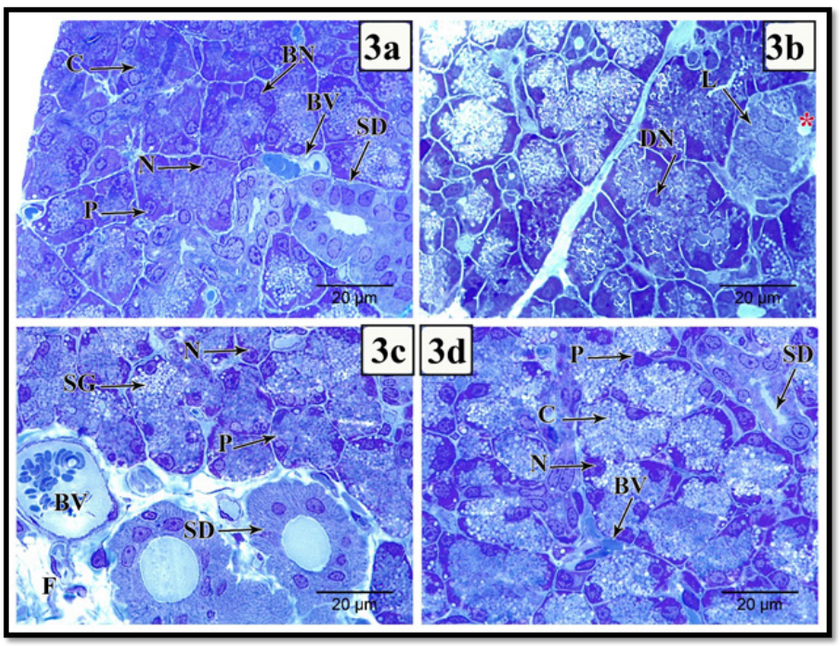

Fig. 3: Photomicrographs of semithin sections in adult rats' parotid glands in the different groups: (3a) The control group shows pyramidal acinar cells (P) having normal nucleus with prominent nucleolus (N) and some cells are Binucleated (BN). The cytoplasm is full of secretory granules (C). The striated ducts are normal (SD) and lined by a single cellular layer. The blood vessels (BV) are normal. (3b) The hypothyroidism-induced group: acinar cells with pyknotic nuclei (DN). The striated ducts are lined with more than one cellular layer (hyperplasia) (L) around a well-defined small lumen (red Asterix) (3c) In the hypothyroidism – induced + ALA group, the acinar cells become pyramidal in shape (P) with a normal nucleus and a prominent nucleolus (N). The striated ducts are lined with a single layer of cells (SD) The blood vessels show minimal congestion (BV). 3d) The ALA- group shows pyramidal acinar cells (P) having normal nucleus with prominent nucleolus (N). The cytoplasm is full of secretory granules (C). The striated ducts are normal (SD) and lined by a single cellular layer. The blood vessels (BV) are normal. (Toluidine blue, x 20 µm, X 1000).

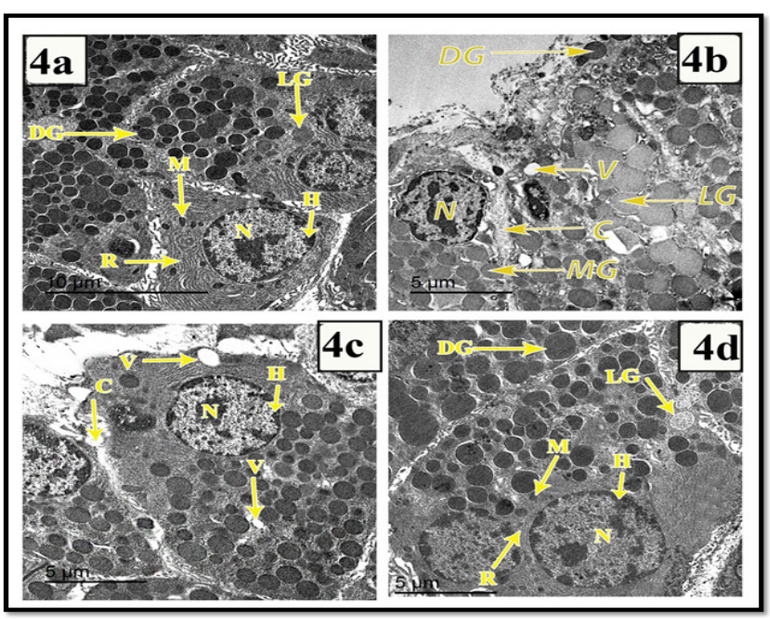

Fig. 4: (4a) An electron micrograph of an ultra-thin section in an adult albino rat parotid gland control group shows: acinar cells with eu-chromatic rounded nuclei (N) , a consistent nuclear membrane and a peripheral thin rim of heterochromatin (H), regular rough endoplasmic reticula (R), mitochondria (M), highly electron dense (DG) and electro-lucent (LG) secretory granules.(4b) An electron micrograph of hypothyroidism-induced group shows: An acinar cell with an irregular nuclear and cell membrane (N). The cytoplasm contains highly electron dense (DG), moderately electron dense (MG), and electro-lucent (LG) secretory granules. Wide intercellular canaliculi (C) and small electron lucent vacuoles (V) are noticed. (4c) An electron micrograph of hypothyroidism-induced + ALA group shows: acinar cells with regular rounded nuclei (N) and a peripheral thin rim of heterochromatin (H). Few cytoplasmic vacuoles (V) are seen. The intercellular canaliculi are still dilated (C). (4d) An electron micrograph of ALA- group shows: acinar cells with eu-chromatic rounded nuclei (N), a consistent nuclear membrane and a peripheral thin rim of heterochromatin (H), regular rough endoplasmic reticula (R), mitochondria (M), highly electron dense (DG) and electro-lucent (LG) secretory granules. (Fig. 4a: TEM, scale bar x 10 µm × 800 ×17) (Figs. 4b, 4c &4d: TEM, scale bar x 5 µm × 1000 ×17)

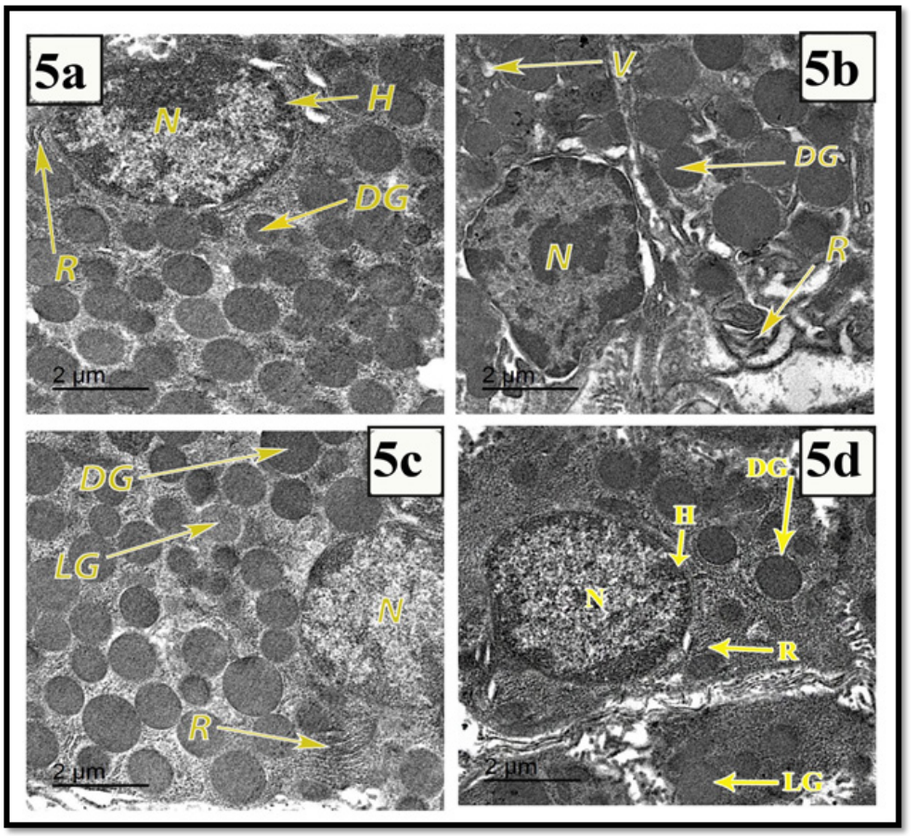

Fig. 5: (5a) An electron micrograph of control group shows: an acinar cell with an euchromatic nucleus, a regular nuclear membrane (N) and a peripheral thin rim of heterochromatin (H). Highly electron dense granules (DG) and regular a rough endoplasmic reticulum (R) are seen. (5b) An electron micrograph of hypothyroidism-induced group shows: an acinar cell with an irregular heterochromatic nucleus and irregular cell membrane (N). The cytoplasm contains highly electron dense granules (DG), a dilated disarranged rough endoplasmic reticulum (R) and vacuolations (V). (5c) An electron micrograph of hypothyroidism-induced+ ALA group shows: an acinar cell with a regular rounded nucleus (N), highly dense (DG) and electro-lucent (LG) secretory granules, and a non-dilated highly developed rough endoplasmic reticulum (R). (5d) An electron micrograph of control group shows: an acinar cell with an euchromatic nucleus, a regular nuclear membrane (N) and a peripheral thin rim of heterochromatin (H). Highly electron dense (DG) and electro-lucent (LG) granules, regular a rough endoplasmic reticulum (R) are seen. (TEM, scale bar x 2 µm, × 2000 ×17)

Table 1: Biochemical assays for all experimental groups

Parameters	Control	Alpha Lipoic acid (ALA)	Hypothyroidism- Induced	Hypothyroidism- Induced + Alpha Lipoic Acid	*P-value*
T3	1.260±0.168	1.330±0.075	0.183± 0.015[ab]	1.130±0.141[c]	0.0001*
T4	4.136± 1.010	4.356±1.359	2.516± 0.513	3.473±1.081	0.2073
TSH	30.430±1.746	30.176± 1.028	39.966±1.342[ab]	31.203±2.296[c]	0.0002*

(n=9)
a $p<0.05$ in comparison with control b $p<0.05$ in comparison with ALA c $p<0.05$ in comparison with Hypothyroidism- induced group

Table 2: Body, Parotid Gland and Relative Weight for All Experimental Groups

Parameters	Control	Alpha Lipoic acid (ALA)	Hypothyroidism- Induced	Hypothyroidism- Induced + Alpha Lipoic Acid	*P-value*
Parotid weight	0.256±0.036	0.249± 0.025	0.139±0.014[ab]	0.191±0.025[abc]	0.0001*
Body weight	282.00±19.23	276.00±16.35	205.00± 11.75[ab]	255.20±10.93[abc]	0.0001*
Relative weight	0.090± 0.010	0.090±0.007	0.075±0.007[ab]	0.068±0.016[ab]	0.0001*

(n=9)
a $p<0.05$ in comparison with control b $p<0.05$ in comparison with ALA c $p<0.05$ in comparison with Hypothyroidism- induced group

Table 3: Morphometric results for all experimental groups (Diameter and number of ducts and acini, area percent of LC3 positive immune reaction)

Parameters	Control	Alpha Lipoic acid (ALA)	Hypothyroidism- Induced	Hypothyroidism- Induced + Alpha Lipoic Acid	*P-value*
Diameter of acini	30.532±2.09	33.933± 4.18	57.172±5.21[ab]	44.512± 5.34[abc]	0.0001*
Diameter of ducts	67.312±2.722	69.612±3.75	131.92± 3.51[ab]	30.074± 5.92[abc]	0.0001*
Number of acini	120.00±8.74	111.80± 5.71	53.33± 7.58[ab]	72.77± 5.80[abc]	0.0001*
Number of ducts	18.00±1.58	16.00± 1.58	7.22± 1.39[ab]	12.44±1.66[abc]	0.0001*
Area % of LC3	5.435± 2.219	8.461±3.050	36.238± 8.401[ab]	21.420±6.344[abc]	0.0001*

(n=9)
a $p<0.05$ in comparison with control b $p<0.05$ in comparison with ALA c $p<0.05$ in comparison with Hypothyroidism- induced group

DISCUSSION

This research was carried out to evaluate the therapeutic property of ALA on parotid tissue affected by the induced hypothyroidism. As reported in results' section there wasn't any significant change of control in comparison with ALA group, so, both will be represented as a control group in discussion section.

Animal models where used the hypofunctional status of the thyroid gland was done by giving Carbimazole to the experimental animals. The functional evaluation of the thyroid gland was performed according to the histological or serum T3, T4 and TSH changes which was in agreement with Rivkees *et al*. (2010)[22].

The experiment revealed a significant decrease in T3 serum level in hypothyroidism-induced group comparing with the ALA and control groups. A non-significant decrease in T4 hormonal level is observed on comparing hypothyroidism-induced group with the other groups. Also, a significant increase in TSH serum level was observed on comparing hypothyroidism- induced group with the ALA and control groups.

The research outcomes agreed with those of Hashem *et al*. (2020)[23] where a significant decrease in T3, a highly significant reduction in T4, and a highly significant rise in TSH were detected. The observed changes in the research were in alignment with those of Abd Elazeem *et al*. (2016)[6] who stated that the experimental hypothyroidism was associated with a significant reduction in T3 and T4 and an increase in TSH serum levels.

Moreover, Kandir and Keskin (2016)[24] stated that alterations in T4 and TSH serum levels were documented in hypothyroidism and hyperthyroidism.

It was conveyed that hypothyroidism could cause histopathological alterations and disturb the salivary role of the parotid gland[24]. Another research documented that the values of thyroid hormones would be around the standard levels for the conservation of histological configuration of the gland and the physiological persistence of salivation[25]. Hayat *et al*. (2010)[25] who documented those cellular alterations in the parotid tissues might occur due to hypothyroidism adverse effects. The secretory role of the parotid gland was probable with cells in a regularly effective metabolism. The serous acinar cells were definitely affected in hypothyroidism. One of the most important mechanisms causing hyposalivation was serous acinar wasting.

Previous studies presented that the acinar cells' nuclei became smaller, tended to be asymmetrical and were hypochromatic following hypothyroidism[26,27]. It was recognized that the nucleus was large and RNA synthesis was active in the euchromatic nucleus. That ability was also a marker of the cell metabolic activity[28]. In the comparison achieved in a study concerning the ratios of parotid acinar nuclear morphologies of the control and hypothyroidism groups, although a numerical change existed, the difference was distinguished to be non-significant[25].

In hypothyroid rats, a significant reduction in body weight was found. This agreed with Hashem *et al*. (2016)[2] who stated that a decrease in the body weight gain was noticed in both hyper and hypothyroid rats.

Previous researches recorded the presence of enlarged salivary glands in hypothyroid patients[5,29]. In the study of Hayat *et al*. (2016)[5] where hypothyroidism induction in rats was done by using Methimazole 0.02% for 3 weeks, an obvious atrophy in parotid serous acini was observed. Likewise, this study revealed a significant reduction in the parotid gland weight and a significant serous acinar atrophy on comparing hypothyroidism-induced group with the control and ALA groups.

Hayat *et al*. (2016)[5] stated that hypothyroidism caused non- significant changes in submandibular and sublingual glands' weights which might be due to the fact that they were the biggest salivary glands in rats, so the differences in the parotid gland weight could be observed more than the other salivary glands.

This study did not show any statistical differences regarding the biochemical assay results, body and gland weights on comparing ALA and control groups. This was in agreement with results of Nasr El-Din and Abdel Fattah *et al*. (2020)[30] who didn't find statistical differences between the control and Nigella Sativa Oil (NSO) groups in all the evaluated statistical parameters.

On histopathological examination of the experimental groups, this work showed that in Hx &E sections of the control and ALA groups the parotid gland tissue was formed of lobules. Each one had tightly packed serous acini

and striated ducts with a fine network of an interlobular connective tissue. On the induction of hypothyroidism, the acinar cells lost their normal arrangement and showed pyknosis. The cytoplasm of acinar cells showed relatively large vacuolations with a cellular infiltration. Also, the striated ducts and interlobar ducts became dilated, showed hyperplasia of their cellular lining with wide areas of fibrosis around them. The periductal blood vessels were massively congested. This was in harmony with Ayuob *et al*. (2016) and El Dahrawy *et al*. (2021)[31,32] who documented that the acinar cells' nuclei of the hypothyroid group in both parotid and submandibular glands appeared pyknotic, degenerated with a darkly stained cytoplasm. Some nuclei showed pleomorphism.

On ALA use, this research revealed that most of the typical general acinar arrangement of the gland was restored and the cytoplasmic vacuoles in the acinar cells were extremely decreased. The ducts restored their normal epithelial lining and the fibrosis that surrounded the ducts and blood vessels decreased. In alignment with this work findings, it was conveyed that ALA had an ameliorating effect on hypothyroidism-induced toxicity[33].

Another research also reported that ALA produced tubular diameter and germinal epithelial thickness improvements in the testis of hypothyroid rats[34]. Other authors stated that ALA played an important role as a powerful antioxidant, which could change the histological alterations caused by Carbimazole in the testicular tissue of the animal model[10]. Those positive results might be due to the lipophilic criteria of ALA molecule, which crossed the biological membranes easily, so reaching all cellular partitions leading to the improvement in oxidative stress-induced pathologies, including atherosclerosis, metabolic syndrome and diabetes mellitus. Also, ALA had an anti-inflammatory effect which antagonized the inflammatory signs like cellular infiltration and fibrosis[35].

Autophagy plays a fundamental role in cell development and differentiation through still largely uncertain mechanisms[36]. Autophagy comprises the deprivation and reusing of injured or worn-out organelles in the cell through the autophagosome creation; a double-membrane structure that engulfs cytoplasmic material decomposing it by lysosomal action. An efficient autophagy procedure is important for cell survival against multiple stresses. The diminished autophagosome processing may also be the cause of an undesirable cell death and impaired function[37]. Tissue self-renewal has been demarcated as the process by which an organ replaces its loss of cells and salivary glands are of the organs having the ability of fast regeneration[38].

The results of this research conveyed a significant rise in the immunohistochemical expression of autophagy accompanying proteins in Carbimazole induced hypothyroidism group compared with the control and ALA groups.

The use of ALA revealed a downregulation of LC3 expression. This was in accordance with the studies Orhon *et al*. (2021)[39] who reported that there was an increase in the number of LC3 puncta remained throughout the regeneration of the salivary glands tissue. Also, autophagy played a crucial role during the regeneration process of the salivary glands from the dormant salivary glands stem cells. Mareninova *et al*. (2020) verified that GFP-LC3 expression noticeably increased the endogenous LC3-II level in pancreas in experimental pancreatitis[40,41].

Regarding the light microscopic examination of the semi-thin sections in hypothyroidism- induced group the acinar cells had deeply stained nuclei. The number of secretory granules decreased. The striated ducts showed hyperplasia. This was in alignment with the research result of Helal *et al*. (2020)[42] who documented that parotid gland of thyroidectomized rats had deeply stained basophilic acinar nuclei.

On ALA use, this work revealed that most of acinar cells got back their normal pyramidal shape and normal nucleus with prominent nucleolus. Few cells remained having deeply stained nuclei. The cytoplasm became filled with secretory granules. The striated ducts appeared normal with a single layer of cells but still surrounded by a minimal fibrosis. The blood vessels were minimally congested. This agreed with studies documented that ALA could encourage the salivary glands' regeneration and the damage of secretory acinar cells was the main reason of xerostomia after irradiation. ALA restored salivary acinar cells by reducing apoptosis, inflammation and fibrosis[9,42].

Regarding the ultra-structural results of the hypothyroidism- induced group, the acinar cells had an irregular heterochromatic nucleus and an irregular cell membrane. The cytoplasm had highly electron dense, moderately electron dense, and electro-lucent secretory granules. The rough endoplasmic reticulum became dilated and disarranged. Cytoplasmic vacuolations and congested blood vessels were also observed.

This was in line with studies documented the presence of two cell types in thyroidectomized rats: dark and light acinar cells having many secretory granules, thus signifying parotid gland capability to transport proteins into the circulation via its exceptional categorization and secretor paths[43]. The light acinar cells also showed changed shapes of rough endoplasmic reticulum similar to those stated during hormonal defect and cellular trans-differentiation[44]. Those changed forms occurred due to the interaction between the rough endoplasmic reticulum, other organelles and the cytoskeleton as a result of a new protein formation or cell differentiation[45].

On ALA use, the gland showed a restoration of acinar cells' regular round nuclei with a peripheral thin rim of heterochromatin. The cytoplasm contained highly electron dense, moderately electron dense and electro-lucent secretory granules. Most of the rough endoplasmic reticula became non-dilated highly developed. The cytoplasm showed fewer vacuoles in comparison with hypothyroidism-induced group.

It was documented that ALA use could improve the radiation-induced toxicities in many cases. That radio-protective agent exerted its effects through antioxidant, anti-apoptic, anti-inflammatory, and other mechanisms[46,47]. Another study verified that ALA also rescued radiation-induced salivary gland hypofunction in rats[48].

Interestingly, this work showed that the diameters of parotid acini and ducts were significantly increased whereas their numbers were significantly decreased in hypothyroidism induced group in comparison with the other groups. Consistent with these findings, other studies noted that the administration of Propylthiouracil and Methimazole caused a decrease in the number and size of the acini of the salivary glands. In addition, this result came in harmony with studies, which specified that the pancreatic tissue of hypothyroid rats exhibited a shrinkage in the acini and cytoplasmic vacuolation[6,31,49,50,51].

CONCLUSION

In conclusion, this study established that Alpha Lipoic Acid efficiently cured parotid gland lesions that occurred due to hypothyroidism, via its valuable effect on both histological and functional. Although further studies are essential to illustrate the pathways involved in the effects of ALA, it may be a convenient therapeutic choice to protect the parotid gland against injury after thyroidectomy operations.

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CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

حمض ألفا ليبويك له تأثير علاجي على أنسجة الغدة النكافية في نموذج الجرذ الابيض قصورالغدة الدرقية بواسطة LC-3 المانع لموت الخلايا الأوتوفائية (دراسة مناعية كيميائية، مورفومترية، الكترونية وبيوكيميائية)

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الخلفية: قصور الغدة الدرقية هو مشكلة الغدة الدرقية الشائعة يمكن أن تكون ناجمة تجريبيا عن طريق الأدوية المضادة للغدة الدرقية مثل Carbimazole تم تسجيل تاثير قصور الغدة الدرقية على الغدد اللعابية.

الهدف: لتقييم القدرة العلاجية لحمض ألفا ليبويك (ALA) على أنسجة الغدة النكافية في نموذج الفئران المتعرضة لقصور الغدة الدرقية.

المواد وطرق البحث: تم توزيع الفئران إلى: (١) المجموعة الضابطة مقسمة إلى: المجموعة ١: تتلقى جرعة فموية يومية من الماء المقطر لمدة ٣ أسابيع، المجموعة ٢: تتلقى جرعة زيت الذرة ٢ مل / كجم / يوم لمدة ٤ أسابيع، (٢) تلقت المجموعة الناجمة عن قصور الغدة الدرقية جرعة فموية من ١,٣٥ ملغم /كج/يوم كاربيمازول لمدة ٣ أسابيع، (٣) مجموعة الغدة الدرقية-ALA: تلقت جرعة فموية من ١,٣٥mg/kg/day Carbimazole لمدة ٣ أسابيع ثم جرعة الفم ALA ٦٠ ملغ/كج/يوم لمدة ٤ أسابيع أخرى،(٤) ALA المجموعة: تلقى جرعة من ٦٠mg/kg/day ALA شفويا لمدة ٤ أسابيع.

تم قياس مستويات هرمونات الجسم والغدد و T٣ و T٤ و TSH. أجريت دراسة نسيجية احصائية للأنسجة الغدة النكافية.

النتائج: في فئران قصور الغدة الدرقية، كانت خلايا الغدة النكافية مع نواة غير منتظمة، التكلسات السيتوبلازمية والتسلل الخلوي. وأظهرت القنوات التمدد وفرط التنسج. بالمقارنة مع المجموعة الضابطة ، أظهرت فئران قصور الغدة الدرقية زيادة كبيرة في النسبة المئوية للمنطقة الخاصة بالتعبير المناعي LC3 ، وأقطار الحويصلات والقنوات ومستوى TSH مع انخفاض كبير في مستوى T3 ، وأعداد القنوات و الحويصلات. وفيما يتعلق باستخدام ALA، تم تحسين جميع هذه النتائج.

الخلاصة: ALA بمثابة عامل علاجي جيد مما يؤدي إلى تحسن في الميزات النسيجية الغدة النكافية.