Role of rosemary (Rosmarinus officinalis) extract on carbimazole induced alteration in pancreas of adult male albino rat (histological, immuno-histochemicaland ultrastructural study)

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Abstract: Background: Carbimazole is a drug inducing hypothyroidism. It can induce ameliorative effect on some glands as pancreas. Rosemary (Rosmarinus officinalis) is a herbal which is beneficial in the treatment and prevention of numerous diseases. It can be used in food processing as flavouring and spicy agent. **Aim of the work:** To study alteration in the pancreas of adult male albino rat following carbimazole administration and to evaluate the possible role of rosemary in improving these changes. **Material and method:** 40 male albino rats were divided into four groups: group I (control group), group II (rosemary group) received rosmary extract orally at a dose of (10 ml/kg/day), group III (carbimazole treated) included rats treated with carbimazole orally at a dose of 0.1 mg/kg/day & group IV rats administred carbimazole same dose as group II then supplemented byrosemary extract (10 ml/kg/day). The experiment duration was 30 days. The pancreatic specimens were subjected to light, transmission electron microscopic study and immuno-staining using Ki 67 & Insulin. Morphometric study and statistical analysis of the result was done. **Results:** Histologic and histochemical study of pancreatic tissue of group III showed pathological changes, some acinar cells showed degenerated cytoplasm, vacuolation with pyknotic nuclei. Others, showed hyalinization, apparent decrease in zymogen granules with relative widening of interlobar septum. The interlobular septum was thickened with fatty and mononuclear cell infiltration andcontained congested thickened, dilated blood vessels. Increased deposition of collagen tissue around dilated blood vessels, pancreatic ducts and acini was revealed by Masson trichrome stain. Immuno-histochemically, in group III showed nearly negative immunoreactivity for Ki67 in the nuclei of cells of islet of Langerhans and Negative immunoreaction for insulin was detected in most of the cytoplasm of β-cells of islet of Langerhans. Ultrastructurally, some acinar cells nuclei revealed peripheral condensation of chromatin with dilatation of peri-nuclear membrane. Their cytoplasm showed degenerated mitochondria, dilated rough endoplasmic reticulum and depletion of zymogen granules. Some β-cells were depleted, others showed pyknotic nuclei. However, prior supplementation with rosmery provided better histological picture manifested by less cytoplasmic vacuolation and near normal acinar cells. **Conclusion**: Carbimazole induced hypothyroidism could cause histological alteration on pancreatic structure of rat. Prior supplementation with rosmary could ameliorate carbimazole effect on pancreas.

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Keywords: Carbimazole, hypthyroidism, pancreas, rosmary, ultrastructure, acinar cells, Ki 67, insulin immunostain.

1. Introduction:

Carbimazole is (a thionamide drug) a derivative of 3 - carboxethoxy methimazole group which is metabolized in the liver to methimazole. It is the drug of choice in treatment of hyperthyroidism as it induce decrease in the level of thyroid stimulating hormone and serum thyroxin after two, four and six weeks of its administration **(Saker et al., 2012; Abbassy et al., 1997).**

Francis et al., (2008) studied the effect of oral administration of carbimazole in hyperthyroid cats. Meanwhile, some studies reported many adverse effects associated with carbimazole intake such as renal tubular necrosis **(Ali et al., 1995),** acute pancreatitis **(Marazule et al., 2002),** pulmonary haemorrhage and necrotizing glumerulonephritis **(Calanas - Continente et al., 2005). Zaidi et al. (2004)** demonstrated change in thyroid microstructure in newborn after administration of therapeutic dose of carbimazole during pregnancy and lactation. Moreover, **Vilchez at al., (2006)** concluded that, carbimazole can induce relatively life-threatening complications e.g. hepatotoxicity, agranulocytosis and massive cholestatic jaundice.

The mechanism of action of carbimazole is not completely understood. Carbimazole administration might cause oxidative impairment in vulnerable targets as nucleic acid bases in DNA, membrane content of unsaturated fatty acids, protein thiol groups. The cumulative oxidative impairment during the long term induce deterioration effects on cellular vital structures with subsequent development of different adverse effect **(Sies, 1986).** Previous studies provided data for occurrence of oxidative stress by carbimazole

administration depending on dose- and time-of the study **(Vijayakumar and Nalini, 2006).**

Rosmary leaves is a herbal plant usually included in food processing due to it`s spicy and flavoring properties **(Fu et al., 2007).** It constitutes a main source of polyphenolics such as rosemarinic acid, carnosol, carnosic and ursolic acid **(Peng et al., 2007).** Rosmary leaves have numerous biological properties as anti-oxidative **(Cheung and Tai, 2007)**, anti-tumor, anti-HIV and anti-inflammatory property **(Altinier et al., 2007).** It was reported its beneficial effect in the treatment of inflammatory diseases, peptic ulcer, spasmogenic disorders, atherosclerosis, heart disease, anti-rheumatic, expectorant as well as bronchial asthma **(Atsumi and Tonosaki, 2007; El-Mougy and Youssef, 2011).** Moreover, Rosmary contains carnosic acid which represent a prophylaxis against some liver carcinogens as aflatoxin A **(Costa et al., 2007).**

Previous researches have demonstrated rosemary protective effect in naphthaline nephrotoxicity **(El-Shreif and Issa, 2015)** and had a direct Protective property in motor neuron against acrylamide toxicity in the developing spinal cord in mammals **(Al-Gholam et al., 2016).** Moreover**, El-Mougy and Youssef, (2011)** proved its protective effect against Azathioprine induced pancreatic toxicity in rats. Hence, in this study the possible protective role of rosmary was studied on carbimazole induced alteration in pancreas in a model of male albino rat using histological, immune-histochemical and ultrastructural.

2. Material and methods:

In this study, 40 male Albino rats were chosen, aged 10-11 weeks, their weight ranged between 150-180 gm. The animals were selected from animal house, Faculty of Medicine, Menofyia University (Menofyia, Egypt). Before the experiment, the animals were acclimatized by housing for two weeks in metal cages at standard temperature 25 ^{a} C. They were fed a standard pellet and freely access water.

Drugs and Chemicals:

Carbimazole tablets: Each tablet contained 5 mg of carbimazole (active principle). It was obtained from (CID) Chemical Industries Development Company present in Giza, Egypt. Two tablets were dissolved in 100 ml distilled water to obtain concentration of 0.1 mg/ml.

Rosemary leaves: They were obtained from Chemistry department, Research Center of Agriculture, Cairo, Egypt.

Animal grouping:

In this study, the experimentwas conducted ethically with the guide of protocol recommended by local animal care ethical committee**.**

The rats were randomly divided into four groups, each including ten rats as follow:

Group I: represented control group received distilled water orally in same volume as used to dissolve used drugs for fifteen days.

Group ll: The group which received rosmary extract orally at a dose of (10 ml/kg/day) using gastric tube at same experimental period as group I.

Group III: served as carbimazole treated group. Animals in this group were treated with carbimazole orally at a dose of 0.05 mg/kg/day for a period of 15 days **(Ali et al., 2013; Dakine et al., 2000).**

Group IV: (carbimazole + rosmary group) Rats were orally administered carbimazole treatment same dose as group III for 15 days then received rosmary extract in same dose as group II for another 15 days **(Haloui et al., 2000).**

Carbimazole and rosemary extract were given orally to animals in each group using a curved metal hollow like needle which reach directly to the stomach called as gavage *process* **(Stine and Brown 2006; MacDonald et al., 2004).**

Rosemary extract preparation:

Distilled water was used for washing of rosemary leaves. The leaves were dried in air then powdered. Each 8 gm of rosemary powder was dissolved in 100 ml of distilled water. The resulting solution was boiled for 2 minutes then infused for 10 minutes. The solution was cooled and filtered using filter paper. The obtained solution was given to the rats in a dose of 10 mg/kg/day **(Haloui et al., 2000).**

Biochemical hormonal assay

For determining T3 and T4 level, blood samples were obtained from orbital sinus 15 days after start of experiment for the first three groups. For the last group (IV), sample collection only done after 30 days (period of experiment) in this group. Serum T3 and T4 levels were measured by chemiluminescence immunoassay using Amerlite kits (Bunkyo-ku, Tokyo, Japan) **(Burtis et al., 1994).**

All the animals in each group were weighted before start and at the end of experiment using an electronic balance measured in grams. After rat scarification, the pancreas was extracted from each rat, dried using tissue paper then weighted just before fixation. The pancreas was cut into slices and subjected to the following:

Histological study:

For light microscopic examination, pancreatic specimens were fixed in 10% of formal saline, dehydrated in alcohol, washed in xylol and embedded in paraffin blocks. Using microtom 5µm sections were obtained, stained with haematoxylin and eosin for the general pancreatic architecture and Masson trichrome stain specific for collagen fibers **(Stevens and Wilson, 1996).**

III-Immunohistochemical study:

Immunohistochemical staining was performed following the protocol of manufacturer using avidin – biotin peroxidase method. Both ki 67 and insulin immunostaining were performed. Ki67 immunostain was used to identify cells in active phase of cell cycle while insulin immunostain was used test insulin secretion in b-cells of islet of Langerhans. Ki67 antibody is a mouse monoclonal prediluted antibody (M7248, 1:30; Dako Cytomation, England). Insulin antibody is biotinylated anti-mouse IgG (Dako LSAB kit; Dako, Denmark). Both ki67 and insulin antibodies were used for paraffin embedded specimens. The primary antibody was diluted at 1:30 and 1:100 for ki67 and insulin respectively and was applied on tissue sections for 30 minutes and 1 hour in same previous order. Then, the pancreatic sections were incubated with a mouse monoclonal antibody (secondary antibody). Additionally, the sections were counterstained with haematoxylin and eosin. For ki67, Brown nuclear staining reaction in cells of islet of Langerhans was marked as positive reaction **(Ukropina et al., 2012)** while for insulin, positive reaction was noticed as brown cytoplasmic staining for insulin secreting β -cells of islet of Langerhans **(Andrews et al., 1997)**.

Morphometric and statistical analysis

The mean number of islet area, islet diameter, the percentage of ki67 positive nuclei, insulin positive cells were measured and calculated by using the Leica Q mage system (LICA microsystem corporation, Cambridge, UK). For each parameter, five slides were chosen from each animal. Finally, the mean was calculated for each animal and each group. The calculated data were expressed as mean ± SD. Comparison between the studied animal groups was performed using one-way analysis of variance SPSS program (Chicago, USA), version 17 was used for calculation. P value determined the data significance. Statistically significant P-value was revealed when P was less than 0.05.

Transmission electron microscopy:

Just after animal scarification, pancreatic tail tissue was cut into small pieces about 1 mm^3, fixed in 5% glutaraldehyde for 24 hours, then washed for three to four times in cacodylate buffer (ph 7.3) for 20 minutes for each time. Finally, specimen fixed in osmium tetraoxide for 2 hours, embedded in Epon 812 in gelatine capsules, incubated at 35°C for first day, at 45°C for second day and at 60°C for 3 days **(Bozzola and Russel., 1998)**. Semithin sections (1 µm) thickness were obtained using ultramicrotome. For light microscopic examination, the prepared sections were stained with toluidine blue. Ultrathin sections from chosen areas of blocks were embded in copper grids, contrasted with uranyl acetate and lead citrate and examined under transmission electron microscope (JEOL 100 CX, Tokyo, Japan) at Tanta University, Electron microscope unit.

3. Results:

Biochemical results:

Serum T3 and T4 levels showed significant ($P<0.05$) decrease in group III in comparison with control group. On the other hand, there was non-significant changes in groups II and IV in comparison to group I (control). Moreover, a significant increase ($P<0.05$) in both parameters (T3 & T4) was reported in group II and IV groups as compared to group III (Fig.1 & Table.1).

Pancreatic and body mass weight:

Administration of rat to carbimazole induced a significant decrease in both body and pancreatic weight of animals when compared to the control group ($P<0.05$). however, there were a significant increase in both previous parameters in group IV when compared to group III ($P<0.05$) (Fig.2,3 & Table.2).

Histological results:

H & E stained sections:

Group I (control) showed a normal histological appearance of pancreatic lobules with intervening interlobular septae. The pancreatic tissue composed of exocrine and endocrine portions. The acini represent the exocrine tissue while islets of Langerhans denote the endocrine part. Each pancreatic lobule contained acini. The acinar cells were pyramidal in shape with apical acidophilic and basal basophilic cytoplasm. Islets of Langerhanscomposed of groups of pale stained cells (Fig.4).

Group II (Rosmary treated) had same architecture as control group.

Group III (carbimazole treated) revealed some acini contain pyknotic nuclei with cytoplasmic hyalinizationand multiple vacuolations. The pancreatic lobules were separated by wide interlobular septum with thickening of interlobar septum. Some interlobular septum showed fattyinfiltration with vacuolation of it`sconnective tissue andthickening of blood vessel lining epithelium. In other septal areas, there was proliferation of connective tissue with apparent fibroblastwith nearby localized area of mononuclear infiltration. Some pancreatic ducts were dilated and lined with flattened epithelium while others having irregular outlines with crowded epithelium and hyaline material content (Fig.5).

Group IV (carbimazole and rosmeray treated) showed nearly normal appearance of pancreatic acini, pancreatic duct and islet of Langerhans. (Fig.6).

Masson Trichrome stained sections:

Masson trichrome stained pancreatic sections from Control group revealed thin layer of collagenous fibres in the septa around pancreatic lobules and blood

vessels and around the pancreatic capsule. Rosmary treated group has same architecture as control group. carbimazole treatedgroup showed thick layer of collagenous fibres around acini, pancreatic duct and dilated blood vessels. Carbimazole and rosmeray treated groupdemonstrated relative decrease of collagen fibres around cini, interlobular septum and blood vessel (Fig.7).

Toludine Blue stained sections:

With respect to toluidine blue staining, Control group illustrated pancreatic acini lined by pyramidal cells having basal vesicular nuclei and containing apical secretory granules. Rosmary treated group has same architecture as control group. Carbimazole treated group demonstrated pancreatic acini with degenerated cytoplasm and Pyknotic nuclei with relative decrease of zymogen granules. Note: Dilated congested blood vessel. Islet of Langerhans revealed some cells with pyknotic nuclei. Carbimazole and rosmeray treated grouprevealed relative near normal pancreatic acini with vesicular nuclei and near normal zymogen granules (Fig.8).

Immunohistochemical stains:

Control group showed islet of Langerhans cells with few immuno-positive nuclei for ki67. Negative immunoreaction in the nuclei of cells of islet of Langerhans was detected incarbimazole treated group while incarbimazole and rosmary treated group immuno-positive reaction was noticed in many nuclei of cells of islet of Langerhans (Fig.9).

Positive insulin immunohistochemical staining was demonstrated as brown cytoplasmic staining inβ-cells of islet of Langerhans. Sections of pancreas of control group showedmoderate immuno-positive cytoplasmicreaction. Negative immunoreaction was detected in most of the cytoplasm of β-cells islet of Langerhans incarbimazole treated groupwhile weak positive immunoreaction in someβ cells cytoplasm mean while mild immuno-positive reaction in the cytoplasm of β-cells of islet of Langerhanswas noticed incarbimazole and rosmary treated group (Fig.10)**.**

Electron microscopic results:

Using transmission electron microscopic examination for control group, Acinar cell showed nearly Oval nucleus with euchromatic chromatin and prominent nucleolus. it`s cytoplasm contained some cisternae of rough endoplasmic reticulum near the nucleus, mitochondria andmany elctron-dense secretory granules in the apical part of acinar cytoplasm. Beta cell of islet of Langerhans having euchromatic nucleus with numerous secretory granules in the cytoplasm. Each granule contains an electron dense core encircled by electron lucent halo. The cytoplasm contains mitochondria, cisternae of Golgi apparatus and cisternae of rough endoplasmic reticulum (Fig.11).

Rosmary treated group has same architecture as control group.

In carbimazole treated group, electron microscopic examination of ultrathin pancreatic section revealed acinar cell having irregular nucleus with peripheral condensation of chromatin while some acinar cell had 2 nuclei (binucleated) and others with dilated surrounding peri-nuclear membrane. The cytoplasm showedsome ballooned degenerated mitochondria with ill-defined cisternae, dilated wavy cisternae of rough endoplasmic reticulum withwidely spaced cisternae. Moreover, some acinar cell cytoplasm demonstratedvariable size secretory granules and in other area secondary lysosomes which engulf some cell component with relative depletion of secretory granules, vacuolesandlarge area of collagen accumulation. β cell of islet of Langerhans contained shrunken pyknotic nucleus with peripherally condensed chromatin. The β cell cytoplasm showed dilated cisternae of Golgi apparatus close to the nucleus and variable size secretory granules with some of them devoid of their characteristic halo appearance. Some β cells of islet of Langerhans encircling microphage which contains many variable sized electron dense engulphed secretory granules with microphage pseudopodia extended between cells to engulphit`s content (Fig.12).

Ultrathin section of rosmary and carbimazole treated group revealed nearly normal acinar cell of euchromatic nucleus and It`s cytoplasm having most probably normal mitochondria, parallel shaped rough endoplasmicreticulum cisternae, variable size secretory granules. β cell of islet of Langerhans demonstrated near normal euchromatic neucleuswith its cytoplasm contained near normal mitochondria, secondary lysosome while in other areas of cytoplasmdilated cisternae of Golgi apparatus, variable size normal secretory granules with some of them devoid of their characteristic halo appearance was noticed (Fig.13).

Morphometric study and statistical results

Carbimazole treated group revealed significant decrease in number of islet per pancreatic area and area of islet in group III (carbimazole treated) when compared to group I (control) & II (rosmary treated). Moreover, a significant increase in the previous parameters were detected in group VI (carbimazole and rosmary treated) when compared to group III.

(table. 3)

The percentage of insulin immunopositive cytoplasm and ki 67 immunopositivenuclei were significantly reduced in group III when compared to group I & II. Meanwhile, there were significant increase in immunopositivity for both immunosains in group VI when compared with group II (table. 3 & Fig.14).

Table 1. Serum T3 and T4 in all studied groups (Data were expressed as mean ±SD):

Groups	Serum T3 (nmol/l)	Serum T4 (µg/dl)
Group I (control)	1.07±0.09	13.1±0.75
Group II (rosmary) treated	1.03±0.2	12.5±0.86
Group III (carbimazole) treated	0.56±0.1[a]	6.5±0.31[a]
Group IV (carbimazole and rosmary) treated	0.91±0.19[b]	12.1±1.1[b]

a Comparison between group I & other groups b Comparison between group III & other groups.
c Comparison between group IV & other groups

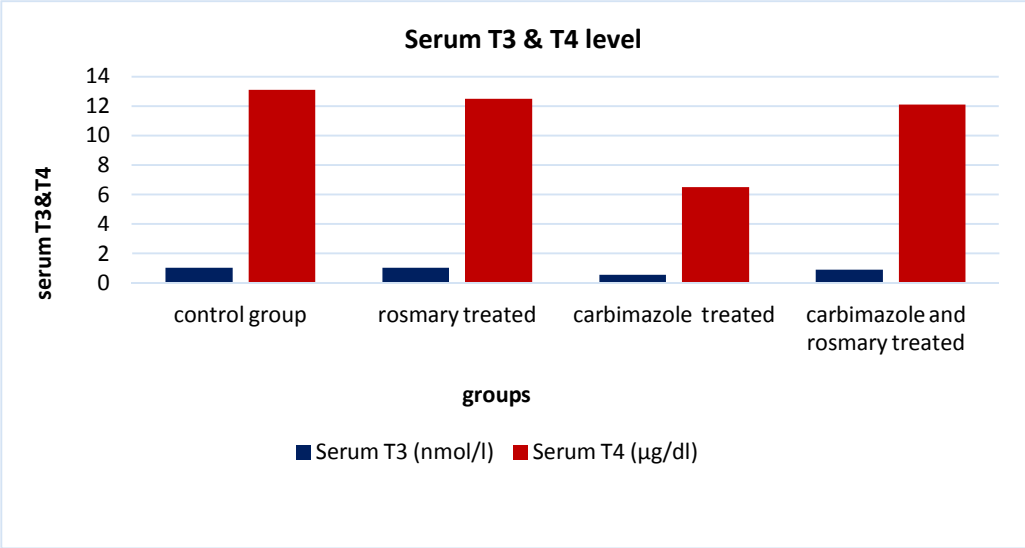


Fig.1 Serum T3 and T4 in all studied groups.

Table 2. Comparison between the values expressed in (mean ±SD) ofrat body weight in gram, pancreatic weight in gram in studied groups:

Groups	**Body weight in gm at start of experiment (mean ±SD)**	**Body weight in gm at end of experiment (mean ±SD)**	**Pancreatic weight in gm (mean ±SD)**
Group I (control)	182±1.2	250±3.7	1.05±0.05
Group II (rosmary) treated	185±2.1	252±4.1	1.1±0.06
GroupIII (carbimazole) treated	186 ±2.4	205±5.1[a]	0.54±0.03[a]
Group IV (carbimazole and rosmary) treated	183 ±2.8	255±4.5[b]	1.15±0.07[b]

a Comparison between group I & other groups b Comparison between group III & other groups.
c Comparison between group IV & other groups

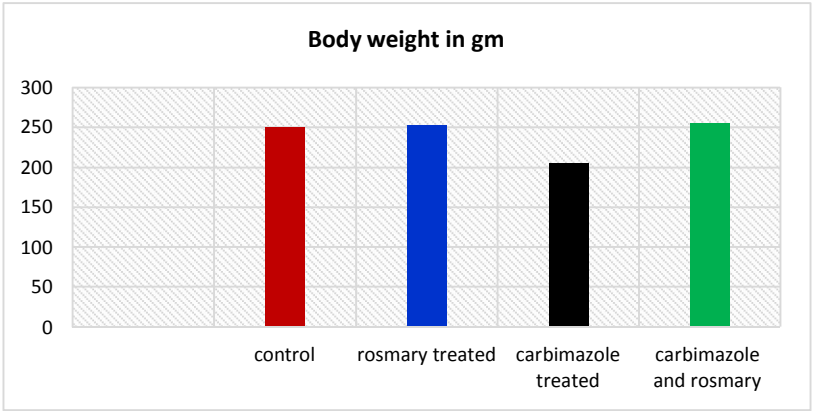


Fig.2. Rat body weight in control and experimental studied animals.

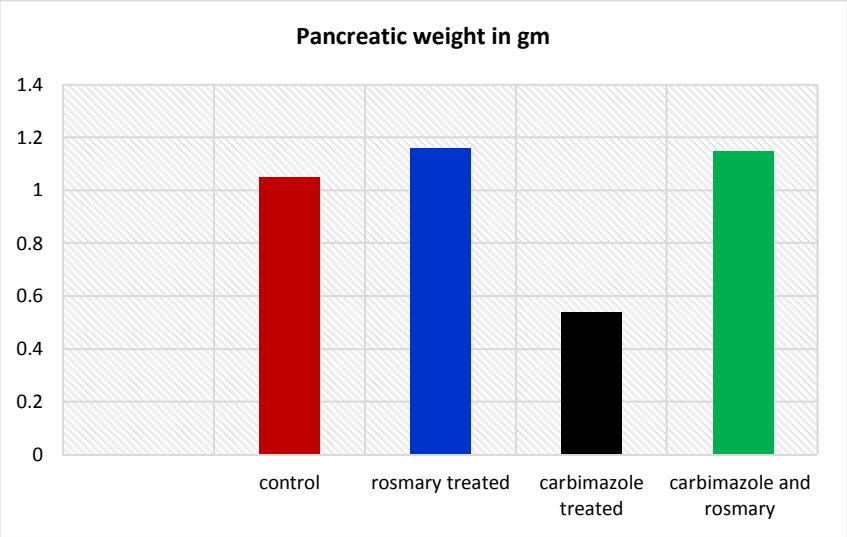


Fig.3. Rat pancreatic weight in control and experimental studied animals.

Table 3. Effect of intake of carbimazole and in concomitant with rosemary on rat pancreatic islet parameters:

Groups	Number of islets/pancreatic area 10 mm²	Area of Islets (mm²)	Insulin positive nuclei (%)	Ki67 positive nuclei (%)
Group I (control)	16.7±0.5	0.0115±0.002	15.7±1.6	10.3±0.9
Group II (rosmary) treated	17.1±0.4	0.0112±0.009	14.9±1.3	10.6±1.05
Group III (carbimazole) treated	6.3±0.9[a,c]	0.0062±0.01[a,c]	6.9±0.79[a,c]	3.4±0.89[a,c]
Group IV (carbimazole and rosmary) treated	15.8±1.5 [b]	0.0106±0.007[b]	13.4±0.9[b]	13.9±1.08[b]

a Comparison between group III & the group I
b Comparison between group III & group IV.
c Comparison between group III & group II.

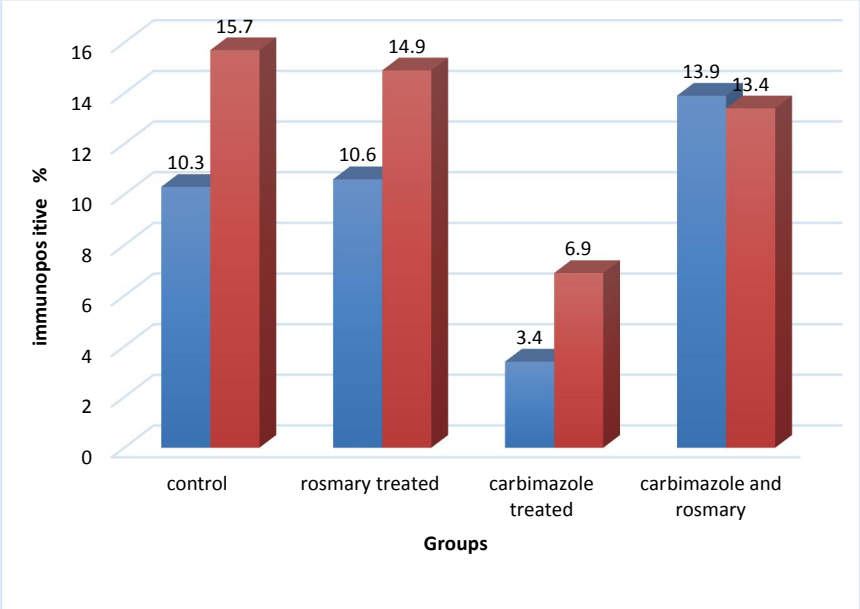


Fig.14. A graph represent immunopositive (ki67 & insulin) in the studied groups

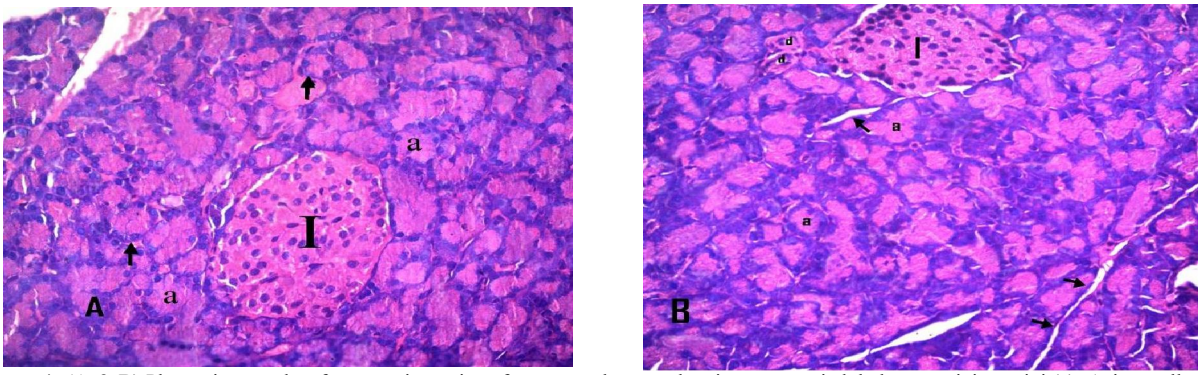


Figure 4: (A & B) Photomicrographs of pancreatic sections from control group showing pancreatic lobules containing acini (a). Acinar cells are pyramidal (thick arrow) with apical acidophilic and basal basophilic cytoplasm. Pancreatic acini are separated by thin interlobular septum (thin arrow) which contain interlobular duct (d). Notice: Islet of Langerhans (I) composed of groups of pale stained cells. **(H & E, x400)**

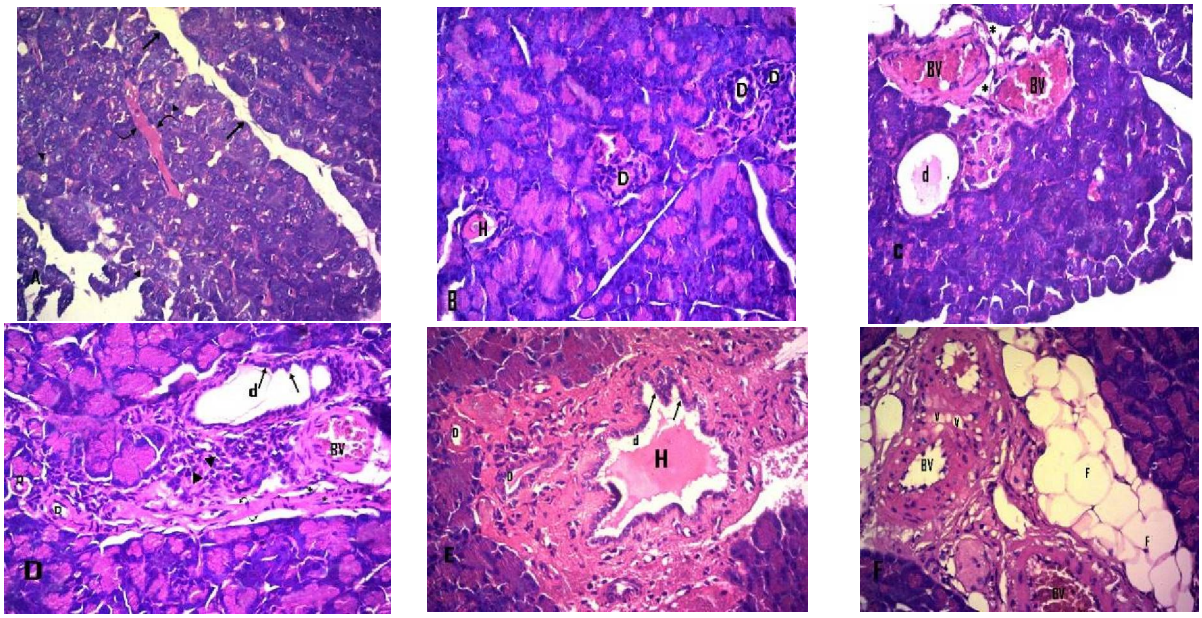


Figure 5: (A-F) Photomicrographs of pancreatic sections from carbimazole treated group showing: **(A)** the pancreatic lobules which are separated by wide interlobular septum (arrow) with thickening of interlobar septum (curved arrow). Notice: some acini contain pyknotic nuclei with multiple vacuolations (arrow head). **(B)** Interlobular pancreatic ducts (D) with proliferated crowded nuclei. Hyalinization can be seen in some acini (H). **(C)** Dilated pancreatic duct (d) lined with flattened epithelium contain hyaline material. Congested dilated blood vessels (BV) with separation of connective tissue septum (*). **(D & E)** Pancreatic duct (d) lined with crowded nuclei with irregular outline (arrow). Hyaline material inside the pancreatic duct (H). Proliferation of connective tissue (curved arrow) with apparent fibroblast (*) with nearby localized area of mononuclear infiltration (arrow head). Notice: multiple interlobular duct (D), dilated congested blood vessels (BV). **(F)** Fatty infiltration of interlobular septum (F) with vacuolation of connective tissue septum. Notice: thickening of epithelium lining of blood vessel. (BV). **(H & E, x400)**

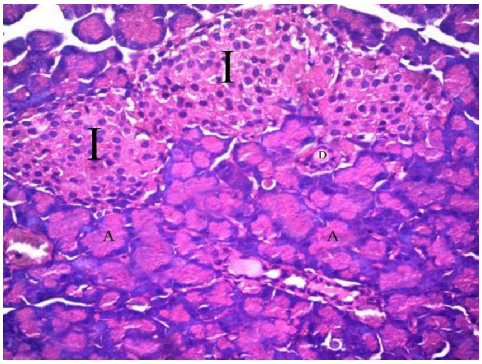


Figure 6: Photomicrographs of pancreatic sections from carbimazole and rosmeray treated group showing nearly normal appearance of pancreatic acini (A), pancreatic duct (D) and islet of Langerhans (I). **(H & E, x400)**

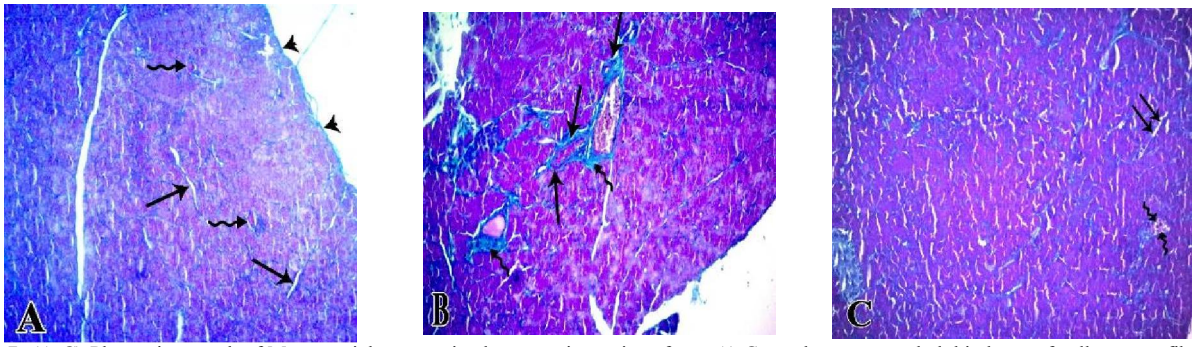


Fig. 7: (A-C) Photomicrograph of Masson trichrome stained pancreatic sections from: **A)** Control group revealed thin layer of collagenous fibres in the septa around pancreatic lobules (thin arrow), blood vessels (corrugated arrow) and around the pancreatic capsule (arrow head). **B)** Experimental group showed thick layer of collagenous fibres around acini, pancreatic duct (thick arrow) and dilated blood vessels (corrugated arrow). **C**) Carbimazole and rosmary treated group demonstrated relative decrease of collagen fibres around cini, interlobular septum (thin arrow) and blood vessel (corrugated arrow). **(Masson trichrome, x 200).**

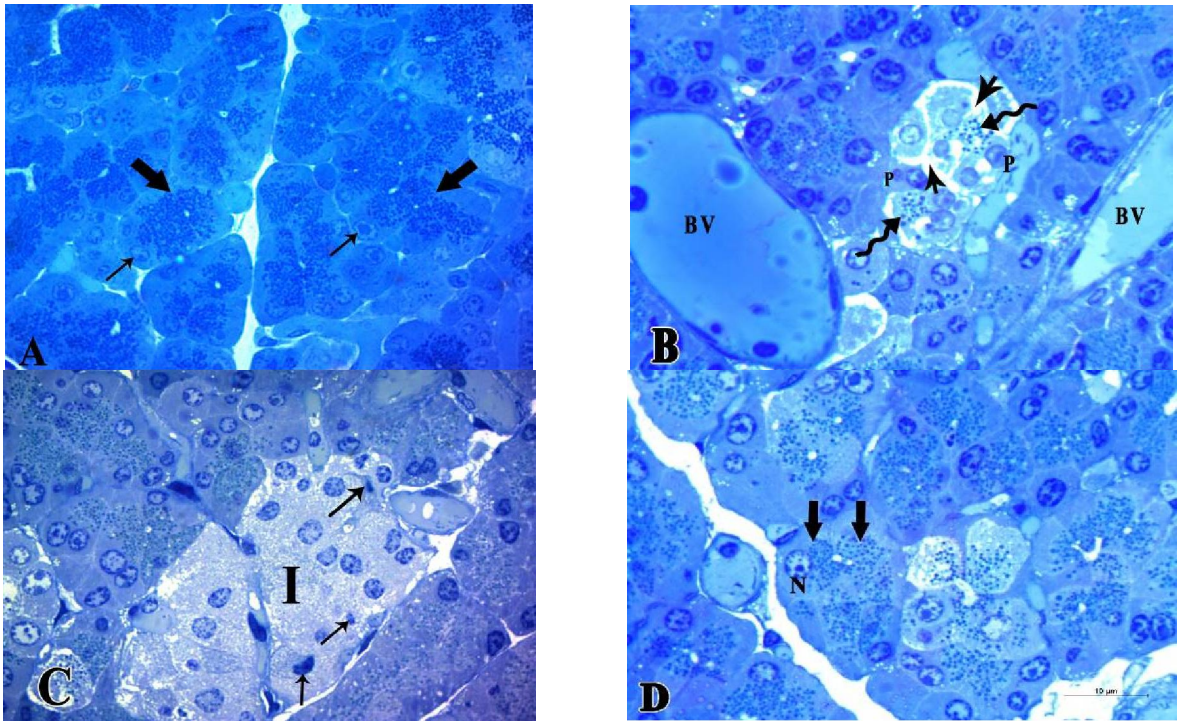


Fig. 8: (A-D) Photomicrograph of toluidine blue stained pancreatic sections **A)** Control group showing pancreatic acini lined by pyramidal cells having basal vesicular nuclei (thin arrow) and containing apical secretory granules (thick arrow)**. B & C)** Experimental group demonstrated pancreatic acini with degenerated cytoplasm (arrow head), Pyknotic acinar nuclei (P) with relative decrease of zymogen granule (corrugated arrow). Note: Dilated congested blood vessel (BV). Islet of Langerhans (I) revealed some cells with pyknotic nuclei (arrow). **D)** Carbimazole and rosmary treated group revealed relative near normal pancreatic acini with vesicular nuclei (N) and near normal zymogen granules (thick arrow). **(Toluidine blue, x1000)**

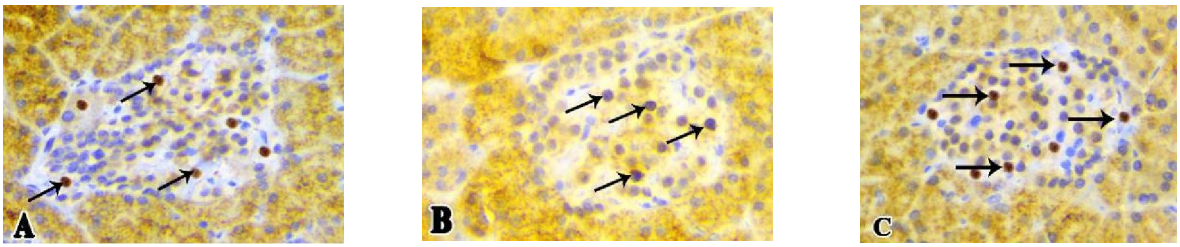


Fig. 9: (A-C) Photomicrograph of ki67 immuno-stained sections from**: (A)** Control group showing islet of Langerhans cells with few immuno-positive nuclei for ki67 (arrow). **(B)** carbimazole treated group showing negative immunoreaction in the nuclei of cells of islet of Langerhans (arrow). **(C)** Carbimazole and rosmary treated group detecting many immuno-positive reaction in the nuclei of cells of islet of Langerhans (arrow). **(Ki67 immunostain, x400)**

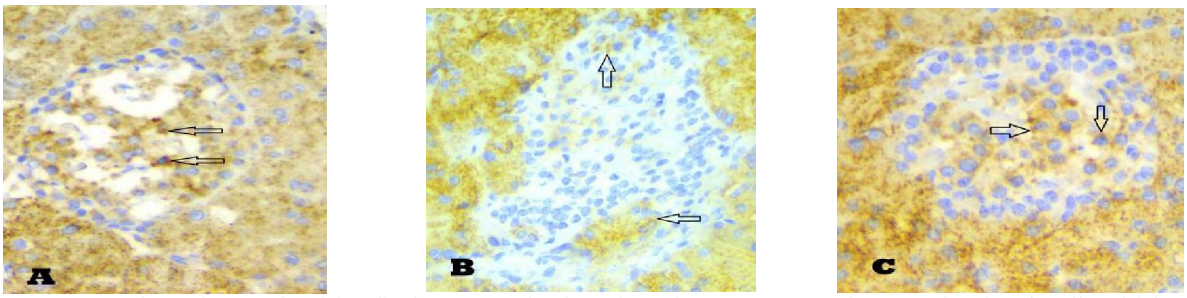


Fig. 10: (A-C) Photomicrograph of insulin immuno-stained sections from: **(A)** Control group showing β cells of islet of Langerhans with moderate immuno-positive cytoplasmic reaction for insulin (arrow). **(B)** Carbimazole treated group demonstrating minimal cells with weak positive immunoreaction in the cytoplasm of β cells of islet of Langerhans (arrow) while most of cells showing negative immunoreaction. **(C)** Carbimazole and rosmary treated group detecting mild immuno-positive reaction in the cytoplasm of β cells of islet of Langerhans (arrow). **(Insulin immunostain, x400)**

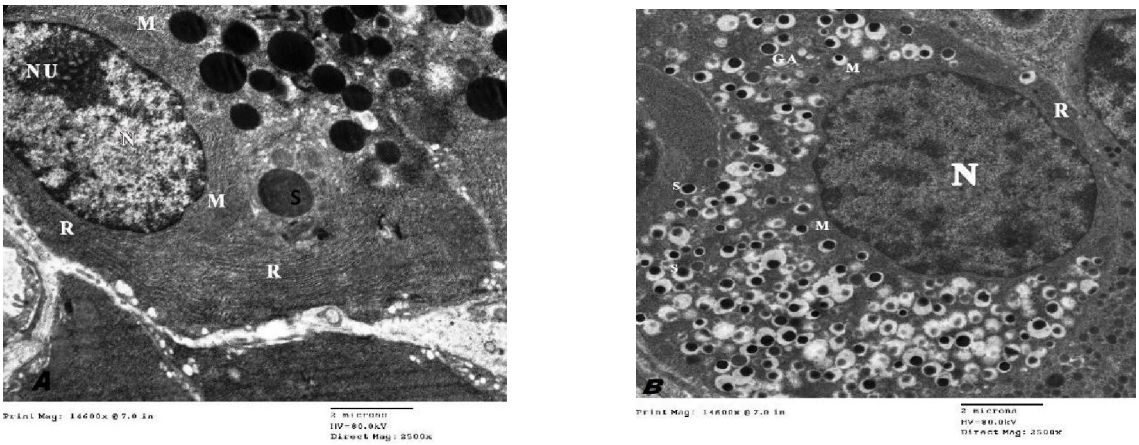
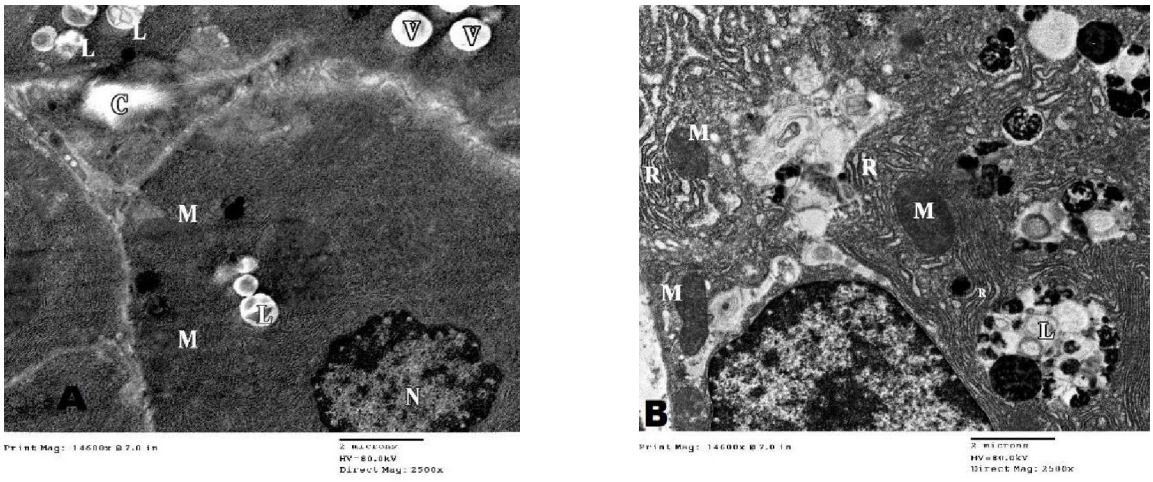


Fig. 11: (A & B) An electron micrograph of: **A)** Acinar cell of control group showing nearly Oval nucleus (N) with euchromatic chromatin and prominent nucleolus (NU), with some cisternae of rough endoplasmic reticulum (R) near the nucleus. Many elctron-dense secretory granules (S) can be seen in the apical part of acinar cytoplasm. Note: Some mitochonria (M) can be detected. **B)** Beta cell of islet of Langerhans of control group having euchromatic nucleus (N) with numerous secretory granules in the cytoplasm. Each granule contains an electron dense core encircled by electron lucent halo (S). The cytoplasm contains mitochondria (M), cisternae of Golgi apparatus (GA) and cisternae of rough endoplasmic reticulum (R) **(TEM, x 14600)**



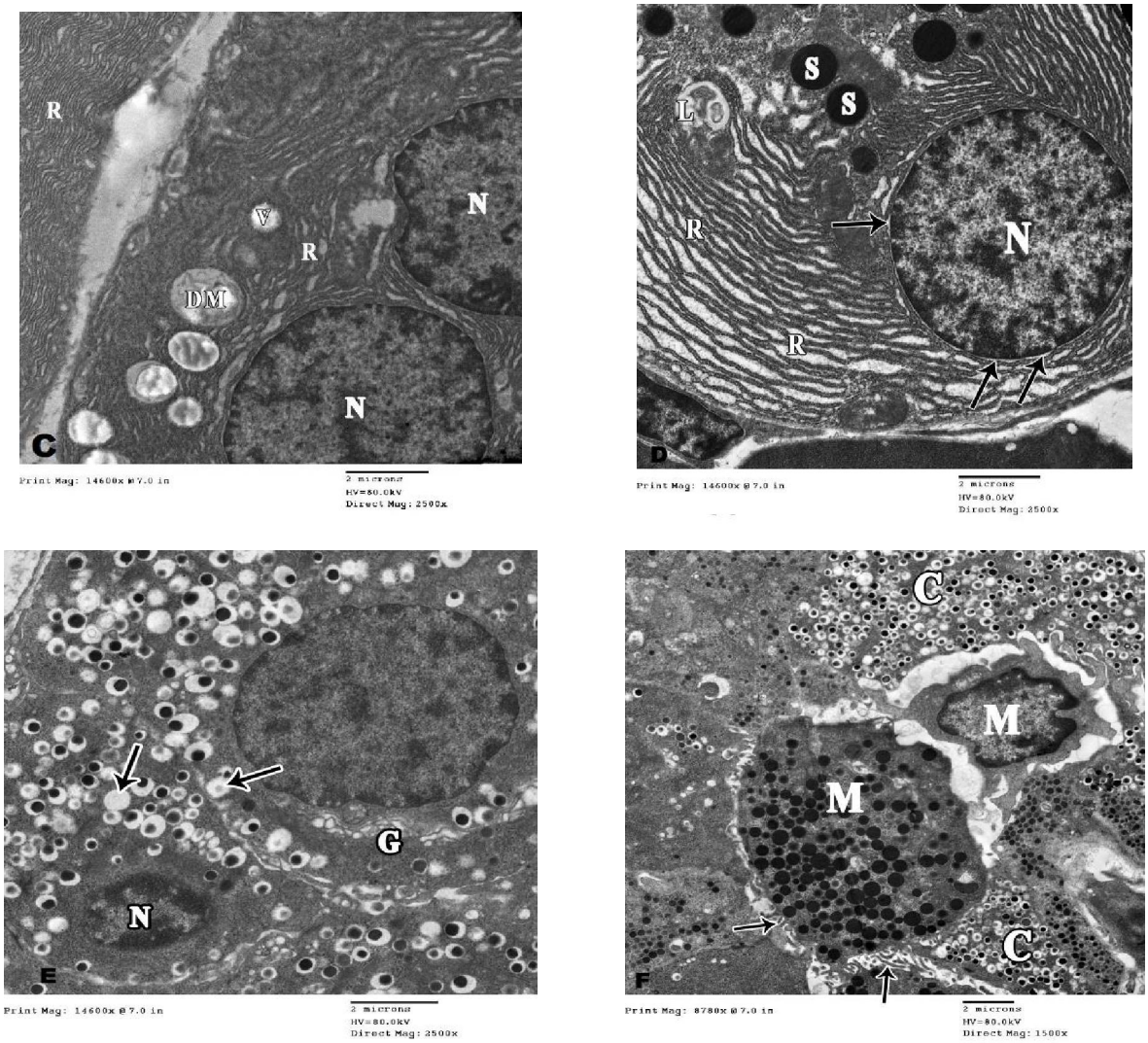


Fig. 12: (A-F) Electron micrographs of carbimazole treated group showing **A)** Acinar cell having irregular nucleus (N) with peripheral condensation of chromatin. The cytoplasm showing vacuoles (V) and secondary lysosomes (L) with relative depletion of secretory granules. Notice some mitochondria (M) and large area of collagen accumulation (C). **B)** Acinar cell having dilated cisternae of rough endoplasmic reticulum (R), mitochondria (M) and secondary lysosomes (L) which engulf some cell component. **C**) Binucleated acinar cell (N) showing some ballooned degenerated mitochondria with ill defined cisternae (DM) with cytoplasmic vacuoles (V). Notice: dilated wavy shape rough endoplasmic reticulum cisternae (R). **D)** Acinar cell demonstrating secondary lysosomes (L), dilated widely spaced rough endoplasmic reticulum cisternae (R) and variable size secretory granules (S). Notice: Nucleus (N) with dilated surrounding peri-nuclear membrane (arrow). **E)** β cell of islet of Langerhans of carbimazole treated group demonstrating shrunken pyknotic nucleus (N) with peripherally condensed chromatin. The β cell cytoplasm showed dilated cisternae of Golgi apparatus (G) close to the nucleus. Notice: variable size secretory granules with some of them devoid of their characteristic halo appearance (arrow). **F)** some β cells of islet of Langerhans (C) encircling microphage (M) which contains many variable sized electron dense engulphed secretory granules. Notice: microphage pseudopodia (arrow) extended between cells to engulph it`s content. (**From A-E TEM, x 14600) & (F TEM, x 8980).**

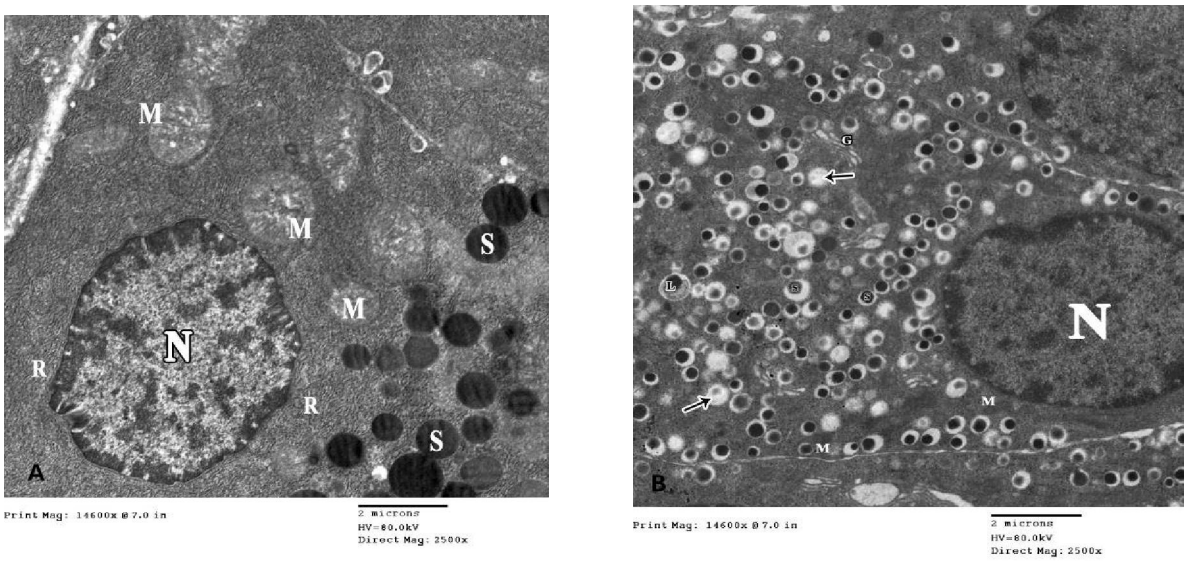


Fig. 13: An electron micrograph of rosmary and carbimazole treated group demonstrating **A)** nearly normal acinar cell of euchromatic nucleus (N). It`s cytoplasm having most probably normal mitochondria (M) and variable size secretory granules (S). Notice: nearly normal parallel shaped rough endoplasmic reticulum cisternae (R). **B)** β cell of islet of Langerhans having near normal euchromatic neucleus (N). The β cell cytoplasm showed near normal mitochondria (M), secondary lysosome (L) and still dilated cisternae of Golgi apparatus (G). Notice: variable size normal secretory granules (S) with still some of them devoid of their characteristic halo appearance (arrow) **(TEM, x 14600).**

4. Discussion:

Carbimazoleis a thionamide drugused for treatment of hyperthyroidism **(Saker et al., 2012)**. In the last years, some studies reported many adverse effects associated with carbimazole intake such acute pancreatitis **(Marazule et al., 2002)** & renal tubular necrosis **(Ali et al., 1995).** Hense, in this study the possible protective role of rosmary was studied on carbimazole induced alteration in pancreas in a model of male albino rat using histological, immune-histochemical and ultrastructural.

In the present study, there was a significant reduction in animal body weight in carbimazole treated group (hypothyroid group). This was in accordance with **(Ali et al., 2013; Ukropina et al., 2012)** who concluded that, loss of weight may be attributed to loss of appetite. This was in contradiction with **Jonklass and Nsouli-Maktabi, (2011)** who reported weight gain in their studied group. This contradiction can be explained by different period of studies, species difference and complex relation between body weight and thyroid condition.

Pancreatic tissue of control group shows normal architecture as findings reported by **El-Gamal and Ghafeer, (2012)** who demonstratednormal pancreatic structure during their study and this proved that, studied control animals were healthy.

Hyalinization of acinar cytoplasm with resulting absent apical acidophilia and basal basophilia was denoted in the present study. This was proved by ultrastructural findings as acinar cell cytoplasmic vacuolation, mitochondrial degeneration and zymogen granules depletion. This agreed with **Blanco-Molina et al., (1991)** findings who reported degenerative changes and concluded to a close relationship between low T4 availability and the resulting morphological changes.

In the present study, hypothyroid acinar secretory cells showed relative decreased numberof secretory granules in some areas and depletion in other area. This phenomenon can be explained by rule of thyroid hormones in transcriptional control of exocrine pancreatic cellular secretion **(Burgi, 1986)** with resulting interference with secretory protein synthesis in pancreas. **Schmidt et al., (2000)** concluded that decreased zymogen granules may be due to impairment in the synthesis of submembranous matrix of zymogen granules.

In this work, in carbimazole treated group, widen interlobular septum between pancreatic lobule with thickening of interlobar septum, Some interlobular septum showed fatty infiltration with vacuolation of it`sconnective tissue andthickening of blood vessel lining epithelium was reported using light microscope. The similar findings was demonstrated by **Aliet al.,**

(2013) who explained tissue oedema due to increased production of hyaluronic acid with it`s hydrophilic property.

In the current study, mononuclear infiltration found in interlobular septum of carbimazole treated group may be explained by pancreatic damage causing disturbed secretion of digestive enzymes and enhance necrosis factor formation. This may lead to increased production of proinflammatory cytokines with consequent mononuclear inflammatory infiltrate as reported in previous studies **(Mareninova et al., 2006).**

Masson trichrome (MT) stained pancreatic sections of carbimazole treated rats revealed increase deposition of collagen fibersin the septa around pancreatic lobules and blood vessels and around the pancreatic capsule in comparison with control group. This went in harmony with **Taha et al., (2013)** who demonstrated an increaseddepositionof connective tissue around pancreatic acini, blood vessels and ducts in rat model treated with L-asparaginase. Similar histological findings were seen by **Abdul-Hamed and Mustafa, (2013)** who concluded that, deposition of collagen around blood vessels causing vascular insufficiency with subsequent insufficient oxygen reached to the tissue lead to more deterioration and degenerative changes.

Zymogen granules contained inactivated trypsinogen inside which represent the main target for lysosomes inside acinar cells. In hypothyroid cases, activation of trypsinogen occurs inside acinar cells not in the duodenal lumen with resulting cellular destruction (**Kumar et al., 2002).** The cellular damage subsequently enhances free radical production causing cell membrane and DNA damage causing irregular nucleus with peripheral condensation of chromatin and dilated surrounding peri-nuclear membrane.

Accumulation of variable sized vacuoles with contents in acinar cells cytoplasm appeared ultrastructurally as autophagic vacuoles contained degraded material in carbimazole treated group. These findings were in line with **Hirota et al., (2006)** who mentioned these characteristic vacuoles in both human and experimental pancreatitis.

In the present study, ultrastructural examination of carbimazole treated group showed acinar cell cytoplasm contained degenerated mitochondria with loss of it`s cisternae and contained electron- dense material**. Gukovsky et al., (2012**) concluded that, mitochondria have a key role in mediating cell death as permeability of it`s cell membrane can enhance necrosis and apoptosis. It represented a point of irreversible effect ending by cell death.

Utrastructurally, in carbimazole group, there was dilatation in RER tubules and cisternae of Golgi apparatus in acinar cell cytoplasm. This agreed with **Andrzejweska et al., (1998**) who observed same findings in their study on taurocholate induced acute pancreatitis in rats. They explained these changes due to deficient ATP in acute pancreatitis with subsequent insufficient energy supply which lead to morphological changes in RER.

Examination of the β cell cytoplasm ultrastructurally in group III demonstrated dilated cisternae of Golgi apparatus close to the nucleus and variable size secretory granules with some of them devoid of their characteristic halo appearance. These data go with data obtained by **Ukropina et al., (2012)** who attributed reduction in insulin production due to dysfunction of β cells. Moreover, expression of insulin gene is indirectly under thyroid hormones control (**Andrali et al., 2008).** This was proved in the present study by negative expression of insulin immunostin in most of cells of islet of Langehans.

A significant decrease in mean area and number of islet per pancreatic area in group III (carbimazole treated) when compared to group I (control) & II (rosmary treated) was reported in the present study. Moreover, there was reduced immune-expression of ki 67 inislet of Langerhans cell nucleiincarbimazole treated group which indicates cessation of proliferation of cells of islet of Langerhans and reflected also by negative expression of insulin immunostain in same former group.

Rosemary extract have been reported to have antioxidant, antispasmodic **(Peter, 2004)**, antimicrobial **(Issabeagloo et al., 2012),** anticancer, anti-hyperglycemic and anti-inflammatory properties (**Madina et al., 2017**). Our results demonstrated that, rosemary has the ability to act as pancreatic protective agent against carbimazole-induced pancreatic hypothyroidism through it`shistomorphological protective and anti-apoptotic effects. This was apparent by almost near normal architecture of pancreatic acini and islet of Langerhans. This denote, that rosemaryable to the attenuate inflammatory & apoptotic pathway in the pancreatic tissue and protect against pancreatic toxicity in rat.

Administration of rosemary extractinduced it`s protective effects against carbimazole-induced pancreatic damage through its antioxidant properties and decreased lipid peroxidation and hence tissue damage. Rosemary might induce its antioxidant property by it`s probable protectiveability as it inhibitsreactive species attacks tocell membranes due to its high concentration of carnosic and rosmarinic acid which previously reported as natural antioxidants **(Al-Gholam et al., 2016)**.

Moreover, Rosmary has beneficial effect in cancer pancreas, cancer breast **(González-Vallinas et al., 2014),** cancer colon **(Scheckel, et al., 2008**), cancer lung **(Anusuya and Manoharan, 2011)** and

gastric cancer **(Lee et al., 2007)** due to it`s antiproliferative, anti-apoptotic and antioxidant mechanisms with significant inhibition of cell viability and cell transformation in cancer cells.

Recent studies demonstrated valuable effect of rosmary in improving diabetes mellitus in studied group of patients. It also improved anti-oxidant enzymes as Malondhyde (MDA) and Glutathion in diabetic patients **(Madina et al., 2017).** Moreover, **elshreif and Issa, (2015)** reported improvement of renal architecture in naphthaline induced toxicity when administered with rosmary. Additionally,, **Takahashi et al., (2008)** demonstrated that rosemary protected against histopathological changes and oxidative stress induced by doxorubicin in heart, liver and kidney of mice. They explained histopathological and biochemical improvement in the studied animals as rosemary extracts are capable to contribute electrons to reactive radicals, modifying them to more stable reactive species with subsequent blocking them from approaching biomolecules, such as polyunsaturated fatty acids, lipoproteins, amino acids, DNA, sugars and proteins in susceptible biological systems **(Fuhr et al., 2006).**

Conclusion:

Carbimazole causes pancreatic damage in studied experimental rats. Rosmary extract could be administered as protective herbal against long and short term administration of carbimazole to alleviateharmful damaging effects on pancreatic tissue due to it`s beneficial properties as a dietary supplement to hinderthe pancreatic injury and pancreatic disease.

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