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The Possible Protective Effect of Selenium on Pancreas of Adult Male Albino Rats Treated with Bleomycin Sulfate

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ABSTRACT
Background: Pancreas is one of the most vital complex organs in the body. Bleomycin sulfate is a cytotoxic drug that may injure the pancreas. **Objective:** To detect the effect of bleomycin sulfate on pancreas of adult male Albino rats and to assess the possible pancreatic protection by selenium use. **Materials and methods:** This study continued 10 days and included 20 adult male Albino rats that were categorized into three groups: group I served as the control group. Group II: bleomycin sulfate treated group. Group III: bleomycin sulfate and selenium treated group. Pancreatic specimens were excised and processed for light and electron microscopic examination. Furthermore, Anti-insulin immunohistochemical staini was assessed and statistically analyzed. **Results:** Treatment with bleomycin sulfate induced distortion and damage of pancreatic architecture with areas of hemorrhage and necrosis. The acinar cells showed vacuolation of cytoplasm with either euchromatic or pyknotic nuclei. Islets of Langerhans appeared distorted, shrunken with difficult differentiation between beta cells and alpha cells. Most of these changes were improved by selenium administration. **Conclusion:** Selenium has significantly ameliorated bleomycin-induced alterations in the pancreas of adult male Albino rats.
Keywords: Bleomycin sulfate, Selenium, Oxidative stress, Pancreas.

INTRODUCTION
Bleomycin sulfate is a chemotherapeutic antibiotic that attracted interest because it's antineoplastic activity either as a single agent or in combination [(1)]. Bleomycin acts by chelation of metal ions forming single and double strand DNA breaks in tumor cells and hindering the cell cycle with consequent formation of superoxide and hydroxide free radicals [(2)].Antioxidant is a molecule capable of abating or anticipating oxidation of other molecules. Oxygen is a highly reactive atom that is capable of becoming part of possibly harmful molecules called free radicals [(3)].Selenium is a dietary trace element and an antioxidant. It is a constituent glutathione peroxidase (GSH-PX), an enzyme that uses hydroperoxides[(4)]. Selenium exists naturally in a very small concentration and both its deficiency and toxicity are closer than any other trace element [(5)].
This study aimed to detect the effect of bleomycin sulfate on pancreas of adult male Albino rats and to assess the possible pancreatic protection by selenium use.

MATERIALS and METHODS
The experimental animals:
The present study was carried out on 20 adult male Albino rats (average weight 200 -230 g). The animals were kept in a separate cage under standard laboratory and environmental conditions. They were allowed free access food and water.

Ethical approval: this study was conducted in accordance with ethical procedures and policies approved by Animal Care Committee of Faculty of Medicine, Al-Azhar University, Cairo, Egypt.
The "Principles of laboratory animal care" were followed, as well as specific national laws where applicable.

Drugs:
Selenium was purchased as a powder with a trade name (Sodium selenate) from Sigma Chemical Company, Cairo, Egypt. It was dissolved in distilled water and given at a dose of 55 microgram/kg/day orally by gastric gavage for 10 days. The selenium dose was equivalent to the average human therapeutic dose [(6)]. The rat dose was adjusted according to **Paget and Barnes**[(7)]. Bleomycin sulfate was purchased as vial with a trade name (Blenoxane) from Bristol-Myers Squibb Company. It was given as a single daily dose of 2.7mg/kg /day by intramuscular injection for 10 days. This dose was equivalent to the average human therapeutic dose [(8)]. The rat dose was adjusted according to **Paget and Barnes**[(7)].

3-Experimental Design:
Animals were categorized into 3 groups:
Group 1: the control group contained 10 rats which were subdivided into two equal subgroups. Subgroup 1a: (- ve control group) were kept without medication during the whole experimental period (10 days). Subgroup 1b: (Selenium group): each rat received only a single daily dose of selenium for 10 days.

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Group II: bleomycin sulfate treated group contained 5 rats, each rat was received only a single daily dose of Bleomycin sulfate for 10 days.

Group III: bleomycin sulfate and selenium treated group contained 5 rats, each rat received a single daily dose of selenium one hour prior to treatment with bleomycin sulfate for 10 days [9].

4-Histological study:

In all groups, rats were sacrificed under anesthesia and the abdomen was incised at the midline. Afterward, the pancreas was removed carefully and cleaned, pancreatic tissue specimens were obtained from the tail of the pancreas.

1- Light microscopy examination:

Pancreatic specimens were immediately fixed in 10% neutral buffered formaldehyde and processed for paraffin blocks. Then, 5 micrometers thick sections were stained with Hematoxylin and Eosin (H&E), periodic acid- Schiff (PAS) technique and Masson's trichrome stain (MTC) [10].

2- Anti-insulin immunohistochemical Study:

For immunohistochemistry, the tissue sections were deparaffinized in xylene, immersed in 3 per cent hydrogen peroxide to quench endogenous peroxidase activity and microwaved in sodium citrate solution (pH= 6.9) for 15 min for antigen retrieval. The pancreatic tissue sections were incubated with avidin–biotin peroxidase system. The primary antibodies used were mouse monoclonal insulin antibodies (Medico Company, Egypt) at a dilution of 1:100 that incubated with slides for 1 h at room temperature. Then, the sections were counter stained with Meyer's hematoxylin[11].

Morphometric study:

Quantitative morphometric measurements were performed by using software Leica Qwin500 (Leica Microsystems, Switzerland) at Pathology Department, Faculty of Dentistry, Cairo University. The image analyzer was used to measure the area percent of staining. The software masked the selected areas by a blue binary color that could be measured by the computer system. Results were expressed as area percent. The mean ± SD was calculated from the measured area percent of 5 different X100 high-power fields.

Statistical analysis

The data obtained were collected, charted, statistically analyzed and represented graphically. Values were presented as mean, standard deviation (SD) and confidence intervals values. Multiple comparisons testing between groups were done using one-way analysis of variance ANOVA and Tukey's post hoc test for pair wise comparison. The significance level was set at $p \leq 0.05$. Statistical analysis was performed with SPSS 18.0 (Statistical Package for Scientific Studies, SPSS, Inc., Chicago, IL, USA) for Windows.

3 -Transmission electron microscopic examination.

Small pieces of pancreatic tissues were fixed in 2.5% glutaraldehyde for 18–24 h then washed in phosphate buffer (pH 7.4) and post-fixed in isotonic 1% osmium tetroxide then processed. Semi-thin sections were stained with 1% toluidine blue and examined by light microscope. Ultrathin sections were stained with uranyl acetate and lead citrate [12]. Sections were inspected and photographed with a JEOL 1010 transmission electron microscope at the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University, Cairo, Egypt.

RESULTS

1- Light microscopy examination results.

Microscopic examination of H&E stained sections from both the control subgroups **(group I)** revealed the characteristic pancreatic lobules which were separated by thin connective tissue septa with interlobular blood vessels and intraocular ducts. Pancreatic parenchyma was formed of exocrine and endocrine parts. The exocrine (serous) secretory acini were lined by pyramidal acinar cells with basophilic nuclei and acidophilic cytoplasm which filled with apical zymogen granules. The endocrine islets of Langerhans were pale stained rounded or oval areas with well-defined outlines. They were formed of clusters of cells organized in irregular and anastomosing cords separated by blood capillaries. They composed mainly of beta cells (β- cells) occupied the islet core and few peripheral alpha cells (α -cells) **(Figs.1,2)**.

In group II, H&E stained sections revealed distortion of pancreatic architecture with areas of hemorrhage and necrosis. Wide septa in between pancreatic lobules with congested dilated blood vessels and dilated intralobular ducts. The acinar cells showed vacuolation of cytoplasm with either euchromatic or pyknotic nuclei. Islet of Langerhans appeared distorted, shrunken with difficult differentiation between β- cells and α-cells **(Figs.3,4)**.

In group III, H&E stained sections showed marked improvement. The acinar cells and islets of Langerhans appeared nearly similar to those of the control with no vacuolation. β- cells and α-cells were markedly improved as compared to that of the bleomycin treated group. However, the islets of Langerhans appeared to increase in size as compared to the other groups. In addition, the ducts were slightly dilated and the blood vessels were slightly dilated and congested **(Figs. 5,6)**.PAS stained sections in group I revealed normal positive reaction mainly in the pancreatic acini which contained zymogen granules and in the islets of Langerhans **(Fig.7)**. Group II, revealed apparent reduction of the PAS positive reaction in both the acinar

cells and the islet cells **(Fig.8)**. Group IV revealed intense PAS positive reaction in the pancreatic parenchyma **(Fig.9)**. Masson's trichrome stained pancreatic sections showed fine collagen fibers distribution in-between pancreatic acini, around islets of Langerhans and pancreatic ducts wall **(Fig.10)**. In group II marked increase in the collagen fibers deposition was realized in between the acini, around the islet of Langerhans and around the dilated congested blood vessels **(Fig.11)**. In group III mild collagen fibers deposition was observed in between the pancreatic acini, around islets of Langerhans and blood vessels walls **(Figs.12)**.

2- Anti-insulin immunohistochemical results:

Immuno-stained sections in group I showed strong positive reaction for anti-insulin antibodies in the form of dark brown cytoplasmic granules in β- cells **(Fig.13)**. However, in group II there was a marked reduction in the reaction or even loss of reaction in some islets **(Fig.14)**. In group III considerable increase was demonstrated in the reaction in β- cells **(Fig.15)**.

Morphometric results: the mean area percentage of anti-insulin immunostaining in group II was significantly decreased when compared to the control rats. However, group III revealed a significant increase in the mean area percentage versus group II, but had not returned to the normal level when compared to the control group (Table1).

3 -Transmission electron microscopic examination results:

Examination of ultrathin sections of group I revealed that the acinar cell was pyramidal in shape with apical cytoplasm and basal nuclei. The cytoplasm of pancreatic acinar cells was characterized by presence of large number of electron dense zymogen granules. These granules appeared with variable shapes, sizes and densities. The cytoplasm contained rounded or oval mitochondria, well developed rough endoplasmic reticulum (r E R). Each cell had a large basal nucleus bounded by the nuclear membrane. Each cell had an apical surface which was directed towards the lumen of the intra-acinar duct. Intra-acinar duct was found in between the acinar cells with many microvilli projecting in its lumen **(Figs.16,17)**.

In group II, the acinar cell showed marked degeneration with irregularity in their outlines. The zymogen granules were markedly reduced in number and in densities with ballooning of mitochondria and complete destruction of their cristae. The nucleus was irregular in shape, pyknotic with folding and indentation of its nuclear membrane. The intra-acinar duct was dilated with abnormalities in the microvilli projecting into its lumen **(Figs.18,19)**.

In group III, the acinar cell showed obvious signs of improvement and regeneration which were represented by increase the amount and density of the zymogen granules. The rough endoplasmic reticulum and mitochondria appeared nearly normal however, the nuclear membrane was still irregular with indentation at one side **(Figs.20,21)**.

Examination of ultrathin sections of group I revealed the β- cells had cytoplasmic secretory granules which were pleomorphic, rounded in shape and variable in size. These granules contained homogenous dense cores which were separated from the surrounding membrane by wide electron- lucent haloes. These granules contained insulin **(Fig.22)**.

In group II, the β- cells showed multiple signs of degeneration, as marked degranulation of the secretory granules in the form of decrease in amount and density, furthermore some granules appeared empty or vacuolated **(Fig.23)**.

In group III, the β- cells showed notable regeneration with increase in the amount of its secretory granules, but also less than that of the control group **(Fig.24)**.

Table 1: the mean values (± SD) of area percentage of insulin immuno-positive cells/µm².

	Mean of area % ±SD
GI	**12.856±1.761**
GII	**1.435±0.269** [a]*
GIII	**7.041±2.192** [b c]*

*P <0.05= statistically significant.

[a] statistically significant from GI

[b] statistically significant from GII

[c] statistically significant from GI

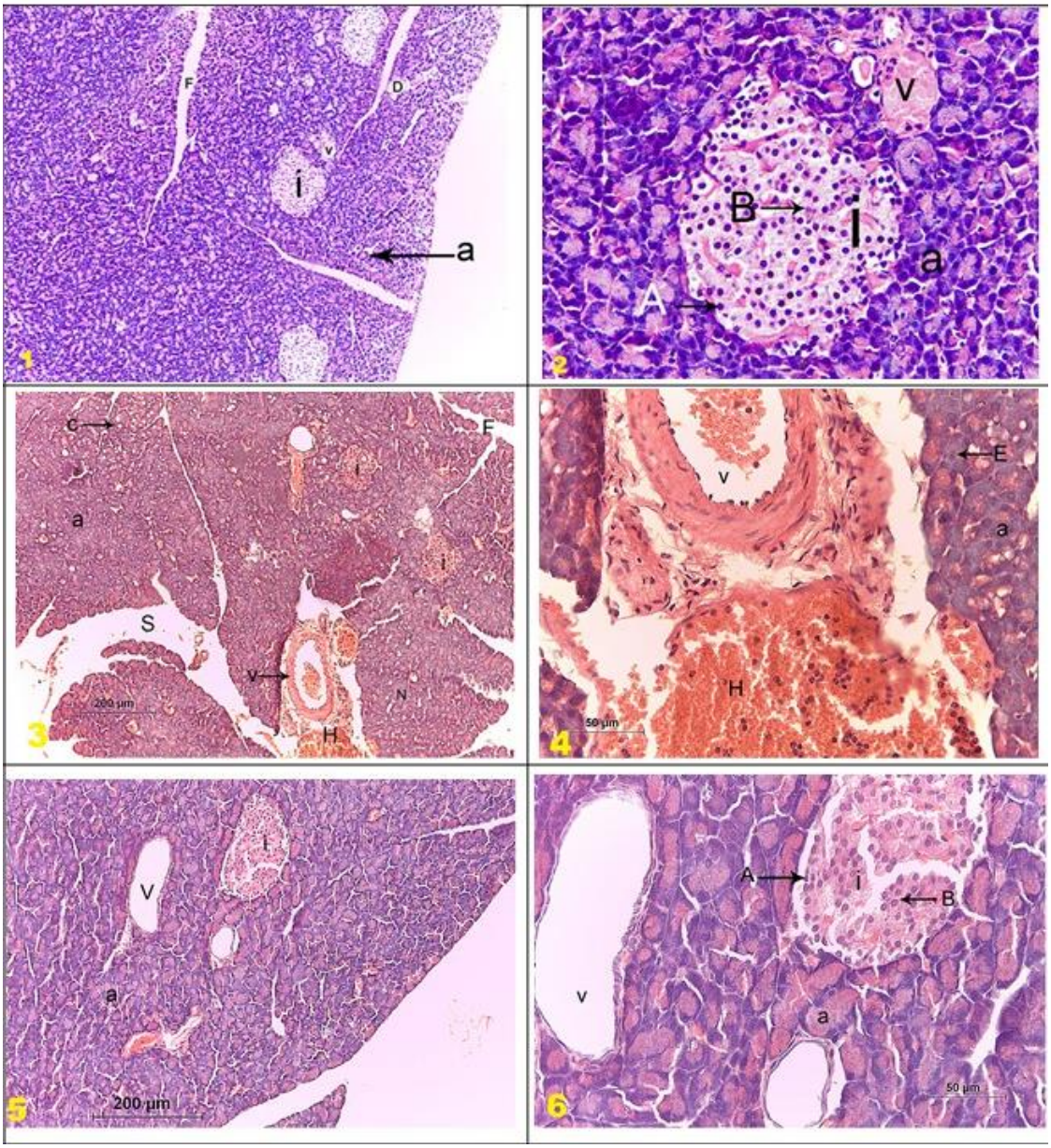

Fig. 1: A photomicrograph of transverse section of pancreas of group I demonstrating thin connective tissue septa (F) acini (a), islets of Langerhans (i), interlobar ducts (D) and blood vessel (v) **(H&E X 100).**
Fig. 2: A photomicrograph of transverse section of pancreas of group I demonstrating acini (a), islet of Langerhans (i), β- cells (B) , α-cells (A) and blood vessels (v) **(H&E X400).**
Fig. 3: A photomicrograph of transverse section of pancreas of group II demonstrating acini (a) vacuolated acinar cells (c), areas of hemorrhage (H), necrosis (N), wide septa (S), shrunken islets of Langerhans (i) and congested and dilated blood vessels (v) **(H&E X 100).**
Fig. 4: A photomicrograph of transverse section of pancrease of group II demonstrating dilated and congested blood vessel (v), hemorrhage (H) vacuolated acini (a) euchromatic nuclei (E) **(H&E X 400).**
Fig. 5: a photomicrograph of transverse section of the pancreas of group III demonstrating nearly normal appearance of the acini (a), islet of Langerhans (i) and dilated blood vessel (v) **(H& E X 100).**
Fig. 6: A photomicrograph of transverse section of the pancreas of group III demonstrating the acini (a), islet of Langerhans (i), β- cells(B) and α-cells (A) and dilated blood vessels (v) **(H&E X 400).**

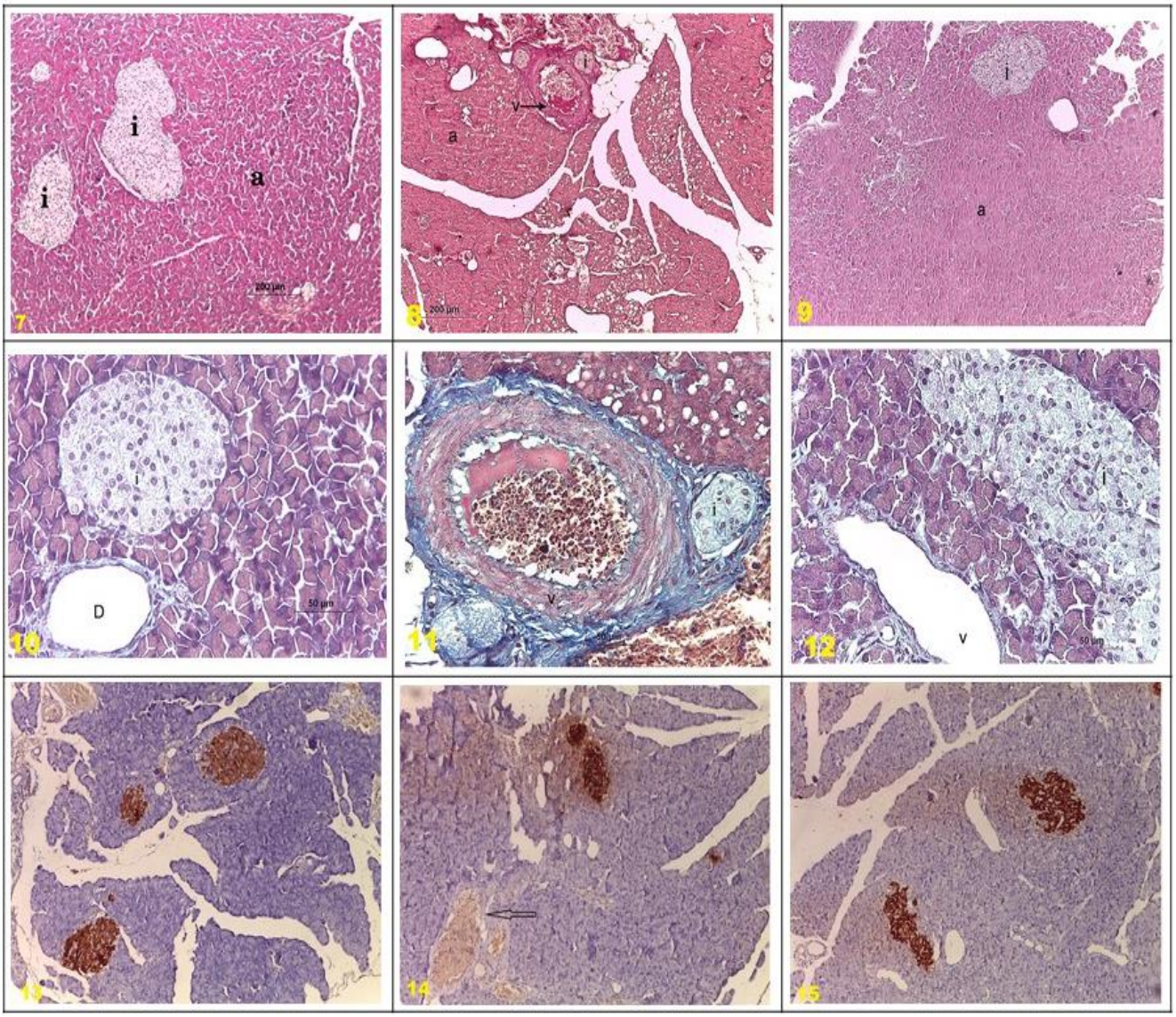

Fig. 7: A photomicrograph of transverse section of pancreas of group I demonstrating PAS-positive reaction in the acini (a) and islets of Langerhans (i)**(PAS X 100).**

Fig. 8: A photomicrograph of transverse section of pancreas of group II demonstrating decreased PAS-positive reaction in both the acini (a) and the islet of Langerhans (i) **(PAS X 100).**

Fig. 9: A photomicrograph of transverse section of pancreas of group III demonstrating a mild PAS-positive reaction in the acini (a) and the islet of Langerhans (i) **(PAS X 100).**

Fig. 10: A photomicrograph of transverse section of pancreas of group I demonstrating normal connective tissue fibers distribution around islet of Langerhans (i) and intralobular duct (D) **(MTC X 400).**

Fig. 11: A photomicrograph of transverse section of pancreas of group II demonstrating increased collagen fibers deposition in walls of the congested dilated blood vessel (v) and surrounding the islet of Langerhans (i) **(MTC X400).**

Fig. 12: A photomicrograph of transverse section of pancreas of group III demonstrating mild collagen fibers deposition around of the islet of Langerhans (i) and the blood vessel (v) (**MTC X 400).**

Fig. 13: A photomicrograph of transverse section of pancreas of group I demonstrating strong positive reaction for anti-insulin antibodies in the cytoplasm of beta-cells **(Anti-insulin Immunostaining X 100).**

Fig. 14: A photomicrograph of transverse section of pancreas of group II demonstrating marked reduction in reaction or even loss of reaction (arrow) **(Anti-insulin Immunostaining X 100).**

Fig. 15: A photomicrograph of transverse section of pancreas of group III demonstrating considerable increase in reaction **(Anti-insulin Immunostaining X 100).**

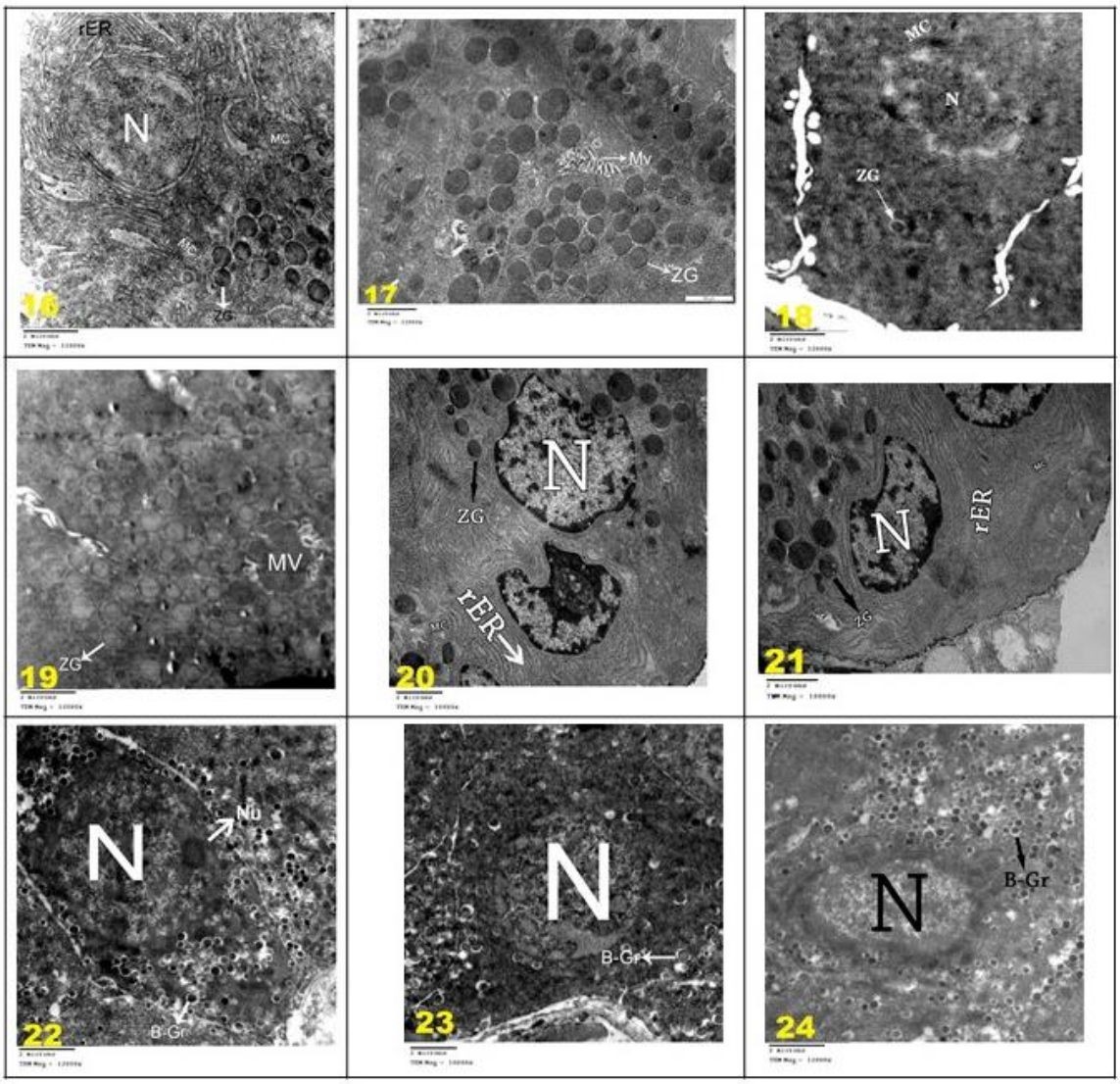

Fig.16: A photomicrograph of ultra-thin section of pancreas of group I demonstrating zymogen granules (ZG), mitochondria (MC), regular rough endoplasmic reticulum (rER) around the nucleus (N) **(X 12000).**
Fig. 17: A photomicrograph of ultra-thin section of pancreas of group I demonstrating zymogen granules (ZG) in the apical part of the acinar cells, microvilli (MV) in lumen of intra acinar duct **(X 12000).**
Figure 18: A photomicrograph of ultra-thin section of pancreas of group II demonstrating the acinar cell with ballooning of mitochondria (MC), decreased zymogen granules (ZG) and nucleus (N) with irregular nuclear membrane **(X 12000).**
Fig. 19: A photomicrograph of ultra-thin section of pancreas of group II demonstrating the acinar cell with marked decrease of zymogen granules density (ZG), dilatation with abnormalities in the microvilli (MV) of the intra acinar duct **(X 12000).**
Fig. (20): A photomicrograph of ultra-thin section of group III demonstrating improvement in zymogen granules (ZG), mitochondria (MC), rough endoplasmic reticulum (rER arrow) around the nucleus (N) with irregular nuclear membrane at one side **(EM. Mag. X 10000).**
Fig. 21: A photomicrograph of ultra-thin section of pancreas of group III demonstrating improvement in zymogen granules (ZG), mitochondria (MC) and rough endoplasmic reticulum (rER) around the nucleus (N) **(X 10000).**
Fig. 22: A photomicrograph of ultra-thin section of pancreas of group I demonstrating, B-cell cytoplasmic granules (B-Gr) nucleus (N) and nucleolus (Nu) **(X 12000).**
Fig. (23): A photomicrograph of ultra-thin section of group II demonstrating, degranulation of β- cells granules (B-Gr) and nucleus (N) **(X 10000).**
Fig. 24: A photomicrograph of ultra-thin section of pancreas of group III demonstrating, notable regeneration of β-cells granules (B-Gr) and nucleus (N) **(X 12000).**

DISCUSSION

Treatment with bleomycin sulfate in this work caused distortion and damage of the pancreatic architecture. Cytoplasmic vacuolation of the exocrine acini and endocrine islet of Langerhans with congestion and dilatation of the blood vessels were realized. These findings are similar to the findings of many investigators who reported vacuolar degeneration in the pancreas after use of many agents, chemicals and drugs [13, 14, 15, 16].

The present results are in accordance with those of **Akbarzadeh *et al.*[13]** who found congestion, vacuolation of serous acini and shrinkage of islets of Langerhans after induction of diabetes by streptozotocin in rat. Also, **Coskun *et al.*[14]** found vacuolation with loss of differentiation between α and β- cells with streptozotocin use. **Coskun *et al.*[14]** suggested that vacuolation might be due to disturbance in the enzymatic secretion system. This suggestion was promoted by **Hirano *et al.*[15]** who claimed that the treatment with cyclosporine induced alteration in the zymogen enzyme systems leading to vacuolation. Ultrastructurally marked degeneration of zymogen granules of the acinar cells with ballooning of mitochondria and nuclear pyknosis were realized. Marked degranulation of the secretory granules of β - cells. These changes are similar to the findings of **Longnecker and Wilson[16]** with another cytotoxic drug (puromycin) in rats and found severe damage to the acini and islets of Langerhans with subsequent acute pancreatitis in rats. In addition, **Johannessen[17]** explained ballooning of the mitochondria due to impaired oxidative phosphorylation inside them. **Johannessen[17]** added that mild degrees of mitochondrial swelling were seen in the reversibly injured cells, while marked swelling were commonly seen in cells near to the point of no return. Ballooning of the mitochondrial membrane was met in dead cells, **Johannessen[17]** claimed that swelling due to the uptake of the calcium by the mitochondria which didn't occur in normal conditions. Moreover, **Marzella and Trump [18]** described the same changes in pancreas of rats treated with cyclosporine which showed abnormalities in the nuclear membrane as an advanced stage in the course of final fate of injured cells.

Furthermore, this study gave an evidence that bleomycin sulfate could produce a picture of moderate to severe form of pancreatitis. This result is in accordance with results of **Carlos *et al.*[19]** who reported that bleomycin sulfate disturbed the normal structure of the pancreas with severe vacuolation of the acini and the islets of Langerhans, dilatations of the blood vessels with extravasation of the blood. **Carlos *et al.*[19]** assumed that these changes might be due to oxidative stress generated by treatment with bleomycin sulfate leading to β- cells injury with subsequent development of type 2 diabetes. Earlier study suggested that the disturbance in the normal state of cells may cause toxic impacts through the generation of peroxides and free radicals that damage all cell components, including proteins, lipids and DNA. Thus, oxidative stress could cause disruptions in normal cell state [20]. In humans, oxidative stress was thought to be included in the development of several diseases or possibly will aggravate their symptoms [21].

However, administration of selenium one hour prior to treatment with bleomycin sulfate led to improvement in architecture of the acini and islets of Langerhans. This is in agreement with the work of **Boussekine *et al.*[22]** who found that the pancreas of diabetic rats received selenium before induction of diabetes was improved in both the acini and the islet of Langerhans. *In vivo* and *in vitro* studies suggested that inorganic selenium mightimprove insulin sensitivity by mediating insulin like actions. Selenium had been shown to decrease the activity of protein tyrosine phosphate, a negative regulator of insulin signal transmission and could possibly reduce insulin resistance [23]. These results can be explained by the ability of selenium to activate glucose uptake and control metabolic processes such as glycolysis, gluconeogenesis, fatty acid synthesis and pentose phosphate pathway and its ability to stimulate a key protein involved in insulin signal cascade [24, 25]. Ultrastructurally, there was an improvement in the zymogen granules and mitochondria in the acinar cells. B-cells showed notable regeneration with improvement in the secretory granules. The present results could be explained in accordance to the results of **Hei *et al.*[24], Becker *et al.*[26]** and **Berg *et al.*[27]** who found that the selenium administration in concomitant with streptozotocin had the ability to stimulate glucose uptake and regulate metabolic processes such as glycolysis, gluconeogenesis, fatty acid synthesis. In addition, selenium had the ability to activate a key enzymes proteins complex involved in the secretion inside the zymogen granules, so the zymogen granules increased in number and in density. In the present study, bleomycin sulfate produced marked depletion in PAS positive reaction which is similar to findings o **Coskun *et al.*[14]** who found the same result after streptozotocin treatment. Moreover, **Pagano and Barazzone-Argiroffo[28]** mentioned that bleomycin sulfate had extensive decrease in glycogen staining by PAS reactions with generalized depletion of glycogen content in the lungs. In the current study, selenium administration revealed improvement in PAS reaction both in acini and the islets. The same result is explained by **Ayhanci *et al.*[29]** who showed similar improvement with selenium administration in the kidneys of rats treated with cyclophosphamide which may be due to the ability of selenium for restoration of gluconeogenesis.

The present work revealed marked increase in the collagen fibers deposition within the parenchyma of the

pancreas and in between the acini by MTC stain. This result can be explained by **Ghosh and Surawanshi**[30] who found reticular changes of the islet as evidence of fibrosis early in diabetic rats. Moreover, **Zhang *et al.***[31] explained this fibrosis which may be resulted from activation of pancreatic stellate cells in response to free fatty acids and lipid peroxidation. Also, **Chandhary *et al.***[32] reported that bleomycin treatment led to over production of reactive oxygen species that can lead to an inflammatory response causing pulmonary toxicity, activation of fibroblasts with subsequent fibrosis. In the present work, selenium reduced bleomycin induced fibrosis. This result can be explained by **Poure*t al.***[33] who related the pathogenesis of pancreatic fibrosis to pancreatic stellate cells. Moreover, immunohistochemically bleomycin produced considerable destruction of beta cell. This result is in accordance with results of **Homo-Delache *et al.***[34] who reported that the islets of Langerhans of pancreas of the diabetic rats were shrunken with necrotic changes in islet cells with subsequent loss of islet architecture. In addition, **Carlos *et al.***[19] found similar result in his work and he explained it by oxidative stress generated by bleomycin sulfate with injury of B-cells. Moreover development of diabetes increased the activity of AMPK (Adenine Monophosphate Protein Kinase) system, start producing a large amount of reactive oxygen species which activate the caspase enzyme and the B-cell gene with subsequent cell apoptosis with affection of the functional parameters of pancreas e.g. glucose transferring factors (GLUTF 1, 2), protein kinase C (PKC), and protein kinase A (PKA). However, the improvement in the percentage area of immunoreactive B-cell with selenium was similar to **Campbell *et al.***[35] who found that the essential trace element selenium regulated pancreatic function. In addition, selenium increased mRNA levels in B-cells and stimulated the increase in both insulin content and insulin secretion in isolated primary rat islets of Langerhans.

CONCLUSION

The present data strongly confirmed the negative role of bleomycin sulfate on the pancreas of the adult male Albino rats. However, pre-treatment with selenium significantly attenuated the bleomycin sulfate -induced injury.

Conflicts of Interest: the authors declared no conflict of interest.

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