Original Article

Effect of experimentally induced diabetes mellitus on the exocrine part of pancreas of adult male albino rat and the possible protective role of Silymarin: light and electron microscopic study

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ABSTRACT

Diabetes is a chronic metabolic disorder that remains a major worldwide health problem. The present study aimed to demonstrate the effect of experimentally induced diabetes on the exocrine part of pancreas and the possible protective effect of Silymarin. Forty adult male albino rats were randomly distributed into four groups (10 rats each). Group I and II (control groups), Group III (diabetic group): the rats received Streptozotocinintraperitoneally once in a dose of 55 mg/kg, and group IV (diabetic group were given Silymarin in a dose of 200mg /kg by oral gavage daily for four weeks). At the end of experimental time, the rats were sacrificed. The pancreas was excised and processed for histological (light and ultrastructural studies) and biochemical examination. Light microscopic examination of pancreatic sections of diabetic rats displayed loss of architecture of pancreatic acini, widening of spaces between acini, dilated interlobular duct, and congestion of blood vessels, excessive collagen fibers deposition around blood vessels and around interlobular ducts. Ultra structurally, the pancreatic sections of diabetic rats showed little secretory granules, widely separated RER, irregular nuclear membrane and clumping of chromatin, fragmented mitochondria, rarefaction, and vacuolation of cytoplasm. Silymarin induction to diabetic rats led to normal architecture of some pancreatic acini but there are wide spaces between them, minimal collagen fibers deposition around acini and around blood vessels. Ultra structurally there were euchromatic nuclei, many secretory granules. Few of the rough endoplasmic reticulum were widely separated. Biochemically GPx and SOD levels in the pancreatic tissues of diabetic rats were significantly lower than the other groups. Treatment with Silymarin for four weeks led to restoration of GPx and SOD to normal level in the pancreatic tissues. The present study demonstrated the pathological effects of induced diabetes on the exocrine part of pancreas and that the use of Silymarin could ameliorate these effects.

Keywords: Diabetes, pancreas, rat, Silymarin.

Introduction

Diabetes is a chronic metabolic disorder that remains major worldwide health problem. Although diabetes has no known cause; complex interplay of several factors including genetic, social, and environmental factors were involved in its etiology [1].

Hyperglycemia, the primary clinical manifestation of diabetes, is the main factor for the development of numerous chronic diabetic complications [2]. hyperglycemia injuries cells by many mechanisms leading to functional changes which are multifunctional and include oxidative stress, non-enzymatic glycation of proteins, increased metabolism of glucose via the sorbitol pathway, greater cholesterol levels and changes in the production of vasoactive substances such as, prostanoids and nitric oxide (NO) and endothelin [3].

Access this article online	
Website:www.japer.in	**E-ISSN:** 2249-3379

How to cite this article: IbrahimLabibAbd Allah, Medhat Mohamed Morsi, MahaKhaledAbd-El wahed, Waleed Mahmoud Ragab, Layla Ahmed Rashed.Effect of experimentally induced diabetes mellitus on the exocrine part of pancreas of adult male albino rat and the possible protective role of Silymarin: light and electron microscopic study. J Adv Pharm Edu Res 2018;8(1):75-81.
Source of Support: Nil, Conflict of Interest: None declared.

Bajaj and Khan[4] reported that there are several sources of reactive oxygen species (ROS) production in diabetes including those of mitochondrial and non-mitochondrial origins; ROS hastens the four important molecular mechanisms tangled in hyperglycemia-induced oxidative tissue damage. These four pathways are activation of protein kinase C (PKC), increased hexosamine pathway flux, increased advanced glycation end-product (AGE), and augmented polyol pathway flux.

Streptozotocin (STZ) is an alkylating agent that has been used frequently to induce diabetes mellitus in animals. STZ causes pancreatic ß-cell death by inducing poly-ADP-ribose synthetase activation, followed by fatal nicotinamide adenine dinucleotide (NAD) exhaustion. STZ moreover impairs the anti-oxidative defense system and increases free radical production [5].

As concerning the effect of diabetes on the pancreatic antioxidant enzymes Gawlik et al, [6] reported that diabetes led to significantly lower levels of glutathione peroxidase and higher levels of glutathione reductase both in plasma and hemolysate. Varsha et al. [7] revealed that diabetes led to significant decrease in the level of superoxide dismutase (SOD), glutathione perioxidase (GPx) and catalase (CAT). In addition, Nurdiana et al. [8] reported that both GSH and SOD activities decreased in the pancreas of diabetic rats, suggesting that pancreatic oxidative stress was increased.

Silymarin is a flavonoid obtained from the milk thistle Silybummarianum.Its protective effects against the oxidative peroxidation in several experimental models and in human hepatic and pancreatic injury has been formerly proved [9, 10]Silymarin working as a free radical scavenger, increasing reduced glutathione (GSH) which functions as a detoxificant of intermediary oxygen reactive products of lipoperoxidation[11].Moreover, Karimi et al. [12]reported that Silymarin increases serum insulin; reduces serum glucose and raises antioxidant enzymes and glutathione. As well as recovers endocrine function and pancreatic morphology in diabetic models. In addition, Kumas et al. [13] reported that Silymarin hinders the entrance of noxious agents into cells by increasing cell membrane resistance.

Several studies pointed out that diabetes led to pathological changes of the exocrine part of the pancreas. Several exocrine acini displayed focal acinar destruction in the form of pyknotic nuclei, cytoplasmic vacuolation, increased collagen fibers deposition around the acini and swelling of the intimal cells of the congested stromal blood vessels [14, 15].

Campbell et al. [16] revealed an unpredicted gathering of neutrophils in the exocrine part of pancreas. These infiltrating neutrophils mostly localized at the level of small blood vessels and to a minor extent nearby to acinar cells. Similarly, the study of Sheweita et al. [17] revealedinflammatory cells infiltrate around the pancreatic duct, disturbed acinar pattern and congested blood vessels.

The aim of current study is to demonstrate histopathological and biochemical changes of the exocrine part of pancreas in streptozotocin-induced diabetes in male albino rat model and to investigate the possible protective effect of Silymarin.

Materials and Methods

Ethical approval

All the ethical protocols for animal treatment were followed and supervised by the animal house, Faculty of Medicine, Cairo University. We followed the guidelines of the ethical standards of the National Institutes of Health guide for the care and use of Laboratory Animals (NIH Publications No. 8023, revised 1978).

This study was performed using 40 adult male albino rats weighing 200-250 gm. The rats were acclimatized in the laboratory for a period of two weeks before carrying out the experiment. They were housed in cages, five rats/cage, under standard laboratory and environmental conditions. The animals were given food and water ad libitum. The rats were divided into four experimental groups (10 rats each).

Group I (Normal control): Received no medications.

Group II (Sham control): The ten rats were divided into 2 groups

- **Group II A:** Received 0.5 ml citrate buffer intraperitoneally once
- **Group II B:** Received 0.4 ml distilled water by gastric tube daily for four weeks.

Group III (diabetic group): Rats received Streptozotocinintraperitoneally once in a dose of 55 mg/kg [18].

Group IV (diabetic group treated with Silymarin): Rats received streptozotocinintraperitoneally once in a dose of 55mg / kg and siylmarin by gastric tube daily at 11 am for four weeks in a dose of 200mg /kg starting 3 days after STZ injection (when rats were confirmed to be diabetic) [9].

Three days after STZ treatment, development of diabetes was confirmed by measuring blood glucose levels in venous blood samples from the rat s tail. Diabetes was confirmed by Ames One Touch Glucometer. Rats with blood glucose levels of 250 mg/dl or higher was considered to be diabetic.

After four weeks the rats were sacrificed by cervical decapitation. The pancreas was excised and prepared for

1- Light microscopic examination:

Pancreatic specimens of each group were fixed in 10 % formalin. Sections of 7 microns' thickness were made and stained with:

Hematoxylin and Eiosin [19] and Masson's trichrome stain [20]

2- Electron microscopic examination: [21]

Electron micrographs from all groups were compared to establish the ultra-structural changes.

3- Biochemical analysis (antioxidant enzyme assay):

Measuring of the level of antioxidant enzymes: Glutathione perioxidase (GPx) and Superoxide dismutase (SOD) in pancreatic tissue of all groups by the method of prasad el al, [22].

4- Image analysis:

The data were obtained by Leica Qwin 500 image analyzer computer system (England). The image analyzer was first calibrated automatically to convert the measurement units (pixels) produced by the image analyzer program into actual

micrometer units. Using the measuring field menu, the area, area % and standard measuring frame of a standard area equal to 118476.6 m^2 were chosen from the parameters in ten fields. In each chosen field of the slides stained by Masson's Trichrome, the pancreatic sections were enclosed inside the standard measuring frame and then the collagen fibres area was measured.

$$\text{Area \% of collagen fibres} = \frac{\text{Area of collagen fibres} \times 100}{\text{Area of standard measuring frame}}$$

5- Statistical analysis:

The obtained data from the image analyzer and the histobiochmeical study were recorded for descriptive statistics and tables. Values were presented as means ± standard error of mean (S. E. M.). One-way analysis of variance (ANOVA) was done for comparison between groups, the significance of the data was determined by P value ($P \leq 0.05$ was considered significant).

Results

1-Light microscopic examination:

Group I and Group II (normal and sham control groups)

Light microscope examination of both normal control and sham control groups revealed that both groups were indistinguishable from each other. Rat pancreas of control groups stained by haematoxylin& eosin showed pancreatic exocrine acini arranged in lobules separated by narrow septa. Acinar cells contain rounded vesicular basal nuclei and apical acidophilic cytoplasm. Endocrine islets of Langerhans appeared as lighter staining areas. The islet of Langerhans looked aswell circumscribed, pale stained, oval or rounded areas within the pancreatic lobules. They were formed of groups of cells arranged in irregular, branching, and anastomosing cords (Fig. 1a).

Sections of rat pancreas stained by Masson's trichrome stain demonstrated fine collagen fibers appear in-between the acini of pancreas, around islets cells, around interlobular duct and blood vessels (Fig.1b).

Group III (diabetic group)

Pancreatic sections of diabetic rats stained by haematoxylin&eosin showed focal affection of the pancreas in the form of loss of architecture of pancreatic acini (Fig. 2c), widening of spaces between acini (Fig 2a&2c), dilated interlobular duct (Figs.2a&2c), and congestion of blood vessels (Figs.2b&2c). Masson's trichrome stain demonstrated excessive collagen fibers deposition around blood vessels and around interlobular ducts (Figs. 3a&3b).

Group IV (diabetic group treated with Silymarin)

Pancreatic sections stained by haematoxylin& eosin showed normal architecture of some acini, loss of architecture of other acini, but there are wide spaces between them and islet cells appeared to be normal (Fig.5a). Masson's trichrome stain showed minimal collagen fibers deposition around acini and around blood vessels (Fig5b).

2-Electron microscopic examination:

Group I and Group II (normal and sham control groups)

Ultra structural examination of acinar cells showed euchromatic nucleus, many electron dense secretory granules, well developed rough endoplasmic reticulum and mitochondria (Fig.1c).

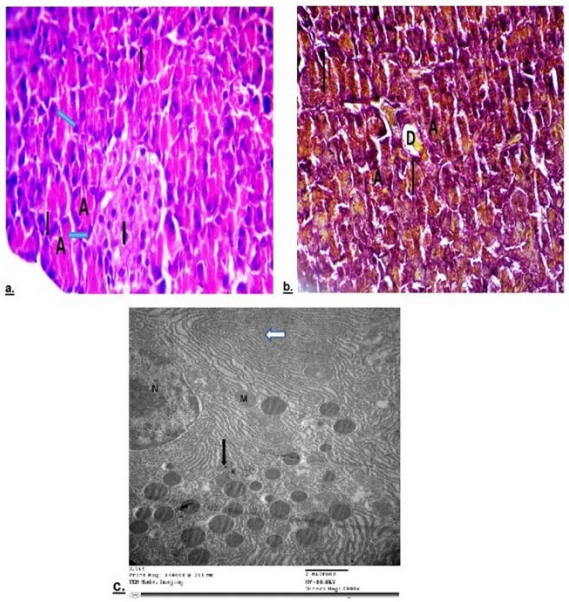

Figure 1: a. photomicrograph of a section of a rat pancreas from control groups demonstrating well circumscribed islet of Langerhans (I) which consists of cells forming cord-like structures. Multiple well-defined rounded or oval exocrine acini (A) are also observed. Pyramidal acinar cells contain rounded vesicular basal nuclei (black arrows) and apical acidophilic cytoplasm (blue arrows) (Hx. &E.x400). B. photomicrograph of a section of a rat pancreas from group I demonstrating very fine connective tissue fibers (black arrow) in-between the acini of pancreas (A) and around interlobular duct (D) (Masson's trichrome x400). c. An electron photomicrograph of a rat pancreas of group II Showing acinar cells having aneuchromatic nucleus (N), well developed cisternae of rough endoplasmic reticulum (white arrow) and numerous electron dense secretory granules (black arrow) and mitochondria (M) (TEM X8000).

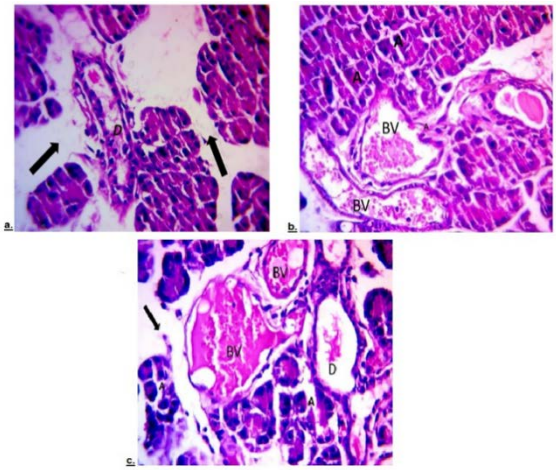

Figure 2: a. photomicrograph of section of a rat pancreas from group III showing dilated interlobular duct (D) and wide spaces between acini (black arrows). b. photomicrograph of section of a rat pancreas from group III showing congested blood vessels (BV) and loss of architecture of acini (A) c. photomicrograph of section of a rat pancreas from group III showing loss of architecture of pancreatic acini (A), congested blood vessels (BV), dilated duct (D) and wide spaces between acini (black arrow) (Hx.&E.x400).

Group III (diabetic group)

Ultrastructural examination showed euchromatic nucleus, little secretory granules and widely separated RER (Fig.4a), irregular nuclear envelop and clumping of chromatin (Fig.4b, 4c & 4d), destructed mitochondria with loss of cristae (Fig.4c) rarefaction and vacuolation of cytoplasm (Figs. 4b&4d)

Group IV (diabetic group treated with Silymarin)

Ultrastructural examination of acinar cells showed euchromatic nuclei, many secretory granules. Some of the rough endoplasmic reticulum were widely separated and others were normal (Fig.5c&5d).

3-Biochemical analysis (antioxidant enzymes assay):

A) Glutathione peroxidase (GPx) level in the pancreas

GPx level in the pancreatic tissues of diabetic rats was significantly lower than the other groups. On the other hand, treatments with Silymarin for four weeks lead to an increase in GPx level to normal level in the pancreatic tissues. Statistical comparison of mean GPx level in different groups, there was statistically significant difference between normal control & diabetic groups, sham control & diabetic groups and between diabetic &diabetic treated with Silymarin groups while there were no significant differences between other groups. P value is significant when less than 0 .05 (table 1).

B) Superoxide dismutase (SOD) level in the pancreas

SOD level in the pancreatic tissues of diabetic rats was significantly lower than the other groups. On the other hand, treatment with Silymarin for four weeks led to an increase in SOD level to normal level in the pancreatic tissues. Comparison of mean SOD level in different groups, there was statistically significant difference between normal control & diabetic groups, sham control & diabetic groups and between diabetic &diabetic treated with Silymarin groups while there were no significant differences between other groups (table 1)

4-Image analysis:

Mean of area % of collagen fibers in the pancreas:

The mean of area % of collagen fibersin pancreatic sections of diabetic rats was significantly higher than the other groups. On the other hand, treatment with Silymarin for four weeks led to decrease in the mean of area % of collagen fibers in pancreatic tissues to normal level (table 1). Statistical comparison of mean of area % of collagen in different groups, there was significant difference between normal control & diabetic groups, sham control & diabetic groups and between diabetic &diabetic treated with Silymarin groups while there were no significant differences between other groups (Table 1).

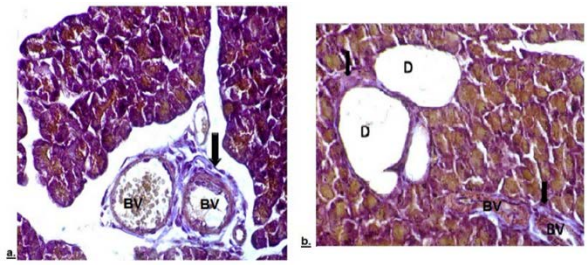

Figure 3: a. photomicrograph of a section of a rat pancreas from group III showing excessive collagen fibers (black arrow) around blood vessels. b. photomicrograph of a section of a rat pancreas from group III showing excessive collagen fibers (black arrows) around ducts (D) and blood vessels (BV) (Masson's trichrome x400).

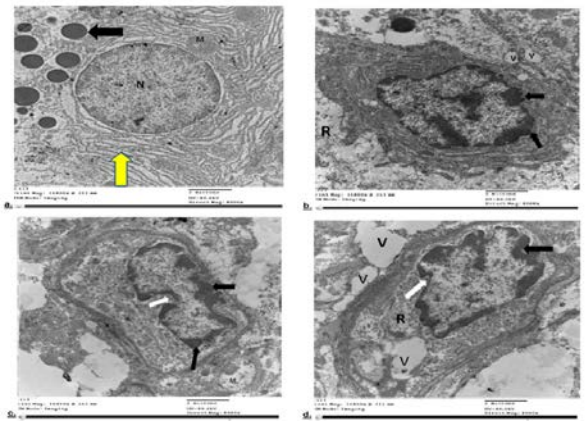

Figure 4: a. An electron photomicrograph of a rat pancreas from group III showing acinar cell having aneuchromatic nucleus (N), widely separated RER (yellow arrow), little secretory granules (black arrow) and apparently normal mitochondria (M). b. An electron photomicrograph of a rat pancreas from group III showing acinar cell having a nucleus (N) with irregular nuclear envelope and clumping of chromatin (black arrows), vacuolation (V) and rarefaction of cytoplasm (R). c. An electron photomicrograph of a rat pancreas from group III showing acinar cell with indentation of nucleus (white arrow), clumping of chromatin (black arrows) also showing destruction of mitochondria with loss of cristae (M). d. An electron photomicrograph of a rat pancreas from group III showing acinar cell with vacuolation (V), indentation of nucleus (white arrow), clumping of chromatin (black arrow) and rarefaction of cytoplasm (R) (TEM x8000).

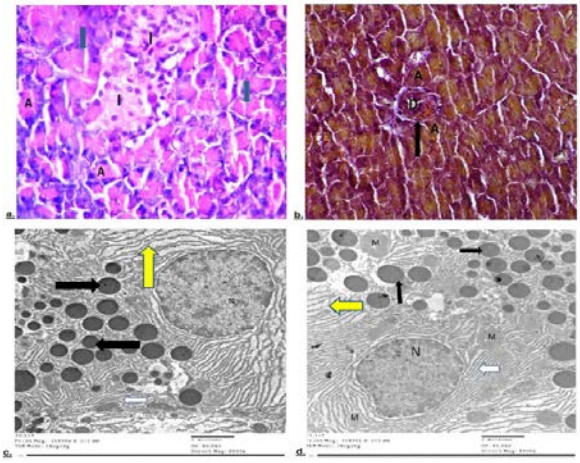

Figure 5: a. photomicrograph of a section of a rat pancreas of group IV showing normal architecture of some acini (A) and loss of architecture of other acini (blue arrows) and islets cells (I) appear to be normal (Hx. &E.x400). b. A photomicrograph of section of rat pancreas from group IV showing minimal collagen fibers (black arrow) around acini (A) and ducts (D) (Masson's trichrome X 400). c. An electron photomicrograph of rat pancreas from group IV showing acinar cell having an euchromatic nucleus (N), many secretory granules (black arrows), Some rough endoplasmic reticulum are widely separated (yellow arrows) and others are normal (white arrow) (TEM x 8000). d. An electron photomicrograph of rat pancreas from group IV showing acinar cell having a euchromatic nucleus (N). Manysecretory granules (black arrows), apparently normal mitochondria (M). Some rough endoplasmic reticulum is widely separated (blue arrow) and others are normal (white arrow) (TEM x 8000).

Table 1: showing the mean glutathione (GPx) and (SOD) level measured in umol/g tissue in the pancreas and the mean area % of collagen, in the different studied groups.

Group	Mean GTH± SD	P-value	Mean SOD± SD	P-value	Mean collagen %± SD	P-value
I(normal control)	59.74 ±12.67		3.66 ±0.43		3.29 ±0.93	
II (sham control)	58.07 ±10.25	0.756* <0.0001*** 0.101****	3.62 ±0.42	0.916* <0.0001*** 0.121****	2.92 ±0.87	0.693* <0.0001*** 0.075****
III (diabetic)	22.69 ±6.15	<0.0001* <0.0001** <0.0001****	1.61 ±0.86	<0.0001* <0.0001** 0.001****	10.24 ±2.56	<0.0001* <0.0001** <0.0001****
IV (diabetic treated with Silymarin)	49.00 ±6.46	0.055* 0.101** <0.0001***	3.01 ±0.72	0.099* 0.121** 0.001***	4.67 ±0.71	0.152* 0.075** <0.0001***

*compared to group 1, ** compared to group 2, ***compared to group 3, ****compared to group 4

. P value ≤ 0 .05 was considered significant.

Discussion

The most common approaches to induce diabetes are established on streptozotocin (STZ) or alloxan (toxic glucose analogs) administration in rodents. They selectively accumulate in the β-cells and result in a marked hypoinsulinemia and subsequent hyperglycemia [23].

In the present study, manifestations of the pathological impact of diabetes mellitus on the exocrine part of pancreas were recorded. Pancreatic sections of rats of diabetic rats stained by Hematoxylin and Eosin showed focal affection of the exocrine part in the form of loss of architecture of pancreatic acini, widening of spaces between acini, dilated interlobular duct and cytoplasmic vacuolation. Other sections revealed congested blood vessels.

The present work supported the work of Attia [24] who reported disturbance in the architecture of pancreatic acini, small vacuoles in the cytoplasm and thickening of the septa between acini. Similar findings were reported by Bera et al. [18] who found that diabetes led to degeneration of pancreatic acini.

The present work coincided with the work of Abdul-Hamid and Moustafa [14] who reported that diabetes led to pathological changes of exocrine part of the pancreas in the form of acinar destruction presented by pyknotic nuclei and cytoplasmic vacuolation.

On the other hand, El-Desouk et al, [25] reported appearance of leucocytic (mononuclear cells) infiltration between acini which was not present in the current work and that may be due to induction of diabetes by alloxan which caused insulinitis.

The present work was in partial agreement with the work done by Sheweita et al. [17] who reported that diabetes caused histological changes in in the form of disturbance of the acini pattern structure, pyknotic nuclei of some acinar cells, vacuolated acini, dilatation, thickening, and congestion of the blood vessels, and inflammatory cells infiltrate around the pancreatic duct.

Nurdiana et al. [8] demonstrated that pancreatic sections of diabetic rats showed swelling of the acinar cells and small vacuoles were observed in almost all acinar cells.

In the current study, histopathological examination of the pancreatic sections of STZ-diabetic rats stained by Masson's trichrome stain revealed increased collagen fibers particularly around the pancreatic ducts and blood vessels. These findings

were in agreement with previous work done by El-Desouk et al. [25] who demonstrated increased collagen fibers deposition around blood vessels. Similar findings were reported by Abdul-Hamid and Moustafa [14] who pointed out that diabetes led to formation of condensed collagen fibers around the acini. Hypertrophy and thickening were noticed in the media with swelling in the endothelium of the intima of the congested blood vessels.

The increase in collagen synthesis in diabetes resulted from activation of pancreatic stellate cells (which have fibroblast like action) in response to increased free fatty acids and lipid peroxidation [26]. Abunasef et al. [27] noticed condensed collagen fibers around some pancreatic ducts and blood vessels. In addition, the later observed that some islets which were completely fibrosed after 6 weeks from the onset of diabetes.

Ultra structural examination of pancreatic sections of diabetic rats revealed acinar cells with rarefied cytoplasm, little secretory granules, widely separated rough endoplasmic reticulum and irregular nuclear envelope with clumping of chromatin. Other sections showed destruction of mitochondria with loss of cristae and vacuolation of cytoplasm.

The present work was in agreement with work of Abdul-Hamid and Moustafa [14] who noticed thatultrastructural examination of the diabetic rats revealed marked changes in pancreatic acini represented by little secretory granules, cytoplasmic vacuolation, destructed mitochondria, autophagic vacuoles and irregular shapes of nuclei.

As regarding GPx and SOD level in the pancreatic tissues of diabetic rats was significantly decreased than other groups. On the other hand, treatment with Silymarin for four weeks leads to an increase in GPx level to normal level in the pancreatic tissues. The present study was in agreement with the work done by Gupta et al., [28]and Varsha et al [7] who revealed that STZ administration led to significant decrease in the level of SOD, GSH and CAT, however treatment with vitamin k1 significantly increase the level of these enzymes to normal level Nurdiana et al. [8] reported that both GPx and SOD activities decreased in the pancreas of diabetic rats, suggesting that pancreatic oxidative stress was increased. In conclusion, the present study demonstrated the pathological effects of induced diabetes on the exocrine part of pancreas and that the use of Silymarincould ameliorate these effects. Therefore, it is recommended to use Silymarin to prevent the side effects of diabetes.

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