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Potential Toxicity of Plant Growth Regulator Gibberellic acid (GA3) on the Pancreatic Structures and Functions in the albino rat

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ABSTRACT

Background: Gibberellic acid (GA3) affects many mechanisms of plant growth including stem elongation by stimulating rapid cell division and elongation, flowering, fruit development and breaking dormancy. GA3 is highly persistent and bioactive in soil for months. Since it is easily absorbed dermally, orally or by inhalation; it can injure liver, kidney, muscle and brain tissues*. Aim of work:* to explore the toxic effect induced by ingestion of residues of GA3 on the pancreas. **Methods:** Sixty male albino rats, were divided into four equal groups: Group I (negative control): received free water. Group II (positive control): received orally 30± 3ml of solution contained 1 N NaOH 5days/week for 6 weeks. Group III (Treated group): received orally daily 2.2± 0.3mg of GA3 5days/week for 6 weeks .Group IV (recovery group): take the same doses and period similar to group III and left without treatment for another 6 weeks. Fasting blood glucose levels were assessed two times per week to all rats during the experiment. At the end of the experimental period, Malondialdehyde (MDA), Superoxide dismutase (SOD) and Glutathione peroxidase (GSH-PX) and Catalase (CAT) were determined, in addition to serum amylase and lipase activity. Moreover, histological examination of the pancreatic tissue was carried by light and electron microscopes further-more, insulin immunohistochemical activity and morphometric study were done. **Results: I**ngestion of residues of GA3 for 6weeks cause significant elevation ($P < 0.001$) of MDA and significant drop ($P < 0.001$) of GSH-PX, SOD and CAT. By stoppage of GA3 lipid peroxidation profile didn't improve completely and still increased above the control levels. Also, GSH-PX and SOD and CAT still decreased significantly. Histological examination express cellular damage with degenerative changes in cells of islet of Langerhans in the form of less population of cells which contained vacuolated cytoplasm and deeply stained or pyknotic nuclei with presence of dilatation of the fenestrated capillary. Ultra structural results of the acinar cells showed irregular contours of nuclei, dilated irregular rough endoplasmic reticulum and reduction of the quantity of the secretory granules with presence of multiple cytoplasmic vacuolations in addition to presence of auotophagic vacuoles. Insulin immunohistochemical staining of islets showing small, atrophied andnegative insulin-immunoreactive spots. The morphometric results represent significant reduction ($P < 0.001$) in the number of islets/pancreas sections and the number of beta cells/islet. The insult which denoted didn't resolve completely by the 6 week recovery period which may suspect the occurrence of chronic pancreatitis due to oxidative stress. **Conclusion:** Residual doses of GA3 exposed the pancreases to oxidative stress and 6 weeks is not enough to complete full recovery.

Keywords: *GA3, Pancreases, oxidative stress, Ultrastructure, Insulin immunohistochemistry, morphometric study.*

INTRODUCTION

Effects of pollutants on the nature became a subject of interest to the majority of the scientists. Also, the effects of these pollutants on human beings, plants and animals were initiated. Plant growth regulators are the most important subject that attract the attention of the investigators among agricultural chemicals.[11]

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Gibberellins are one of the six major classes of plant growth regulators according to the American Society of Agricultural Science.[17]Gibberellic acid (GA3) is one of the most active hormones of gibberellins. It affects many mechanisms of plant growth including stem elongation by stimulating rapid cell division and elongation, flowering, fruit development and breaking dormancy.[31] In nature, ripening is the final stage in the development of a fruit which involves series of physiological and biochemical events leading to changes in color, flavor, aroma, and texture that make the fruits both attractive and tasty.[44] GA3 is widely used in Egypt, to increase the growth of fruits and vegetables.[25]GA3 is highly persistent and bioactive in soil for months. The Environmental Protection Agency has determined its use to be only allowed in low doses. [42]

The World Health Organization listed GA3 as a plant growth regulators related to pesticides. GA3 could possess risk to those professionally exposed, as well as the general population via the consumption of contaminated food products.[45]These chemicals are irritating to the eyes, skin and mucous membrane. Since it is easily absorbed dermally, orally or by inhalation; it can injure liver, kidney, muscle and brain tissues.[12]People may be exposed to residues of GA3 in diet derived from consumption of different types of fruits and vegetables treated with GA3. Exposure to residues may also be through drinking water.[46] Previously, El-Mofty et al. (1994)[15]reported that GA3 has a carcinogenic effect. In animals,chronic GA3 consumption led to increase tumor formation[31] and altered growth hormone levels in the serum, liver and kidney. It also cause an increase in the number of splenic plaque forming cells, circulating white blood cells, hematocrit values and thymus weight in young mice.[12]Madacsiet al.,(1988)[28] and Abd-Elhamidetal.(1994)[2]reported positive influences of GA3 on body weights of rats, poultry, pigs and calves. Also, Gawienowskiet al., (1977)[18] and Gawienowski and Chatterjee (1980)[19]reported that GA3 has number of estrogenic hormone-like actions.

Acute pancreatitis (AP), noninfectious inflammatory disorder of pancreas, not only is the most common cause of hospital admission among gastrointestinal diseases in many countries [36,27], but also is recognized as one of the leading acute diseases worldwide, with rising absolute incidence and age-standardized rates over the past decades.[20] However all the previous experiments clarify the effect of GA3 on the different organs but limited studyexplore its effects on the pancreas. So, the current work was designed to explore the biochemical, histological, ultra structural and immunohistochemical changes induced by digestion of residuesofGA3 on the pancreas of the adult male albino rats and the possible mechanism of such effects.

MATERIALS ANDMETHODS

I-Materials

A) Experimental animal:

60 adult male albino rats (weight 200-220gm) were housed in the animal house in the faculty of medicine for girls Al-Azhar –University, Cairo Egypt, at 21°C–22°C in a 12 hr/12 hr light/dark cycle, fed standard rat chow, and given free access of water. Rats accommodated to the laboratory conditions for 2 weeks before starting the experiment, which conducted according to the guidelines of the Animal Care and Use Committee of National Research Center, Egypt.The rats were divided into four equal groups, of 15 rat/each as following:

Group I (C): received orally free water and served as a negative control.

Group II (C+): received orally 30± 3ml of solution contained 1 N NaOH (1 ml of 1 N NaOH diluted with 1000 ml tap water) 5days/ week for 6 weeks and served as a positive control.[45,12]

Group III (T): Treated orally with daily dose 2.2± 0.3mg of GA3 (which present in 30ml of prepared solution of 75ppm ofGA3) 5days/ week for 6 weeks.[45, 12]

Group IV(R): Treated orally with the same doses and period similar to group III and left without treatment for another 6 weeks and served as a recovery group.

B) Preparation of 75ppm of GA3:

GA3 is white powder produced by Sigma chemical company Egypt. 75mg of GA3 was dissolved in 1 ml of (1 N NaOH) and then diluted with 1000 ml tap water to obtain 75 part per million(75ppm GA3). Since all rats have the same physiologic characters, daily water consumption of each rat in all groups was approximately 30± 3ml which containing 2.2± 0.3mg. of GA3 for each rat per day.[45]

II – Methods:

A) Blood assay for pancreatic functions:

1- Fasting blood glucose were assayed for every rat in all groups two times per week after six hour fasting through all the experimental period. Blood glucose was assayed by the glucose oxidase method, using a glucometer.

2-At the end of the experimental period, 6 weeks, for groups (I), (II), (III) and 12 weeks for group (IV), blood samples were taken from each rat through the retro-ocular puncture, collected in prepared tubes and spinned for the preparation of plasma, which were stored at -20Cº until assayed, then all the studied animals were sacrificed. The abdomens were opened by mid line incision and the pancreases were excised and everyone is divided into two pieces one prepared for light microscopical study and the other for the transmission electron microscopical examination.

B) The biochemical analysis:

a- Malondialdehyde (MDA), a lipid peroxidation product was measured as a marker for oxidative stress in serum,bythe method described by Draper and Hadley, (1990)[14]. The activity of antioxidant enzyme SOD was estimated by colorimetric method described by Nishikimi et al., (1972)[32] and Glutathione peroxidase (GSH-PX) was determined by the method of Rotruck et al., (1973)[38].

b- Serum amylase and lipase activity were evaluated with a spectrophotometric technique by the Olympus AU-2700 auto analyzer(Olympus, Homburg,Germany) using commercial kits(sigma company, Cairo, Egypt)The results were expressed as U/L.

C) Statistical analysis:

To find out the significance of enzymatic changes in groups of the study, data were collected and entered to the Statistical Package for Social Science (IBM SPSS) version 20. Quantitative data were presented as mean, standard deviations and the comparison between more than two groups regarding quantitative data with parametric distribution was done by using One Way Analysis of Variance (ANOVA) followed by Post Hoc analysis (LSD test). The confidence interval was set to 95% and p-value was considered significant at the level of < 0.05 and highly significant at the level of < 0.01.

D) Preparations for histological and immuno-histochemical study:

1- Light microscopy:

Specimens of the pancreases were fixed in Bouin's solution for 48 h. Later, they were dehydrated in graded levels of ethanol, cleared in xylene, and embedded in paraffin wax for sectioning. The 5-µm thick sections were cut, mounted on glass slides, and stained with Haematoxylin and Eosin stain to demonstrate the general histological structure and Masson's trichrome stain to evaluate the collagenous fibers distribution.[7]

2- Insulin immunohistochemistry:

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 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 counterstained with Meyer'shematoxylin.[22]

3- Transmission electron microscopical study:

Specimens which taken from the pancreas of all groups were prepared according to the method of [7], 1–3 mm segment of pancreas were fixed in fresh 3% glutaraldehyde–formaldehyde at -4°C for 18–24 h. The specimens were then washed in phosphate buffer (pH 7.4) and post-fixed in isotonic 1% osmium tetroxide for 1 h at -4°C and then processed. Semi thin sections (1 lm) were stained with toluidine blue. Ultrathin sections (70–80 nm) were stained with uranyl acetate and lead citrate and examined with a JEOL 1010 Transmission Electron Microscope at the

Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University Cairo Egypt.

E) Morphometric study:

Morphometric study was done by the image analyzer computer system Leica Qwin 500 to evaluate:

1- Number of islets/each pancreatic section.

2- Number of Beta cells/islet.

Counting was done at ten fields in each section to count the number of islet (10x). On higher magnification (40x), insulin positive cells in islets were counted randomly on immunohistochemistry.Data were then collected and prepared to the statistical analysis according to Adeyemi et al., (2010).[6]

RESULTS

I - Biochemical results:

The results of the experiment showed that the treatment of rats with Gibberellic acid (75 ppm) caused increased blood glucose level [Table 1] &[Figure 1], in addition to changes in the lipid peroxidation biomarker (MDA) and antioxidant enzymes GSH-PX, SOD and CAT. The major lipid peroxidation product MDA increased significantly in the treated group in comparison with the control groups. By stoppage of GA3 lipid peroxidation profile didn't improve completely and still increased above control levels. Also, GSH-PX and antioxidant enzymes decreased significantly in treated group with GA3 indicating highly significant decrease in the scavenging power of the tissue due to the toxic effect of GA3 which didn't return to control level by cessation of the drug [Table 2] &[Figure 2].

Table 1: Blood glucose level in all the studied groups.

Groups	Blood glucose level
GI(control)	70.87± 0.472
GII (control+)	71.62 ± 0.351
GIII(treated)	174.27± 0.410*
GIV(recovery)	112.92± 0.251**

Values are expressed as mean ± SEM.

*Test of significance between GI (control) and GIII (treated) rats at p < 0.001.

**Test of significance between GIV (recovery) and GI (control) rats at p < 0.001.

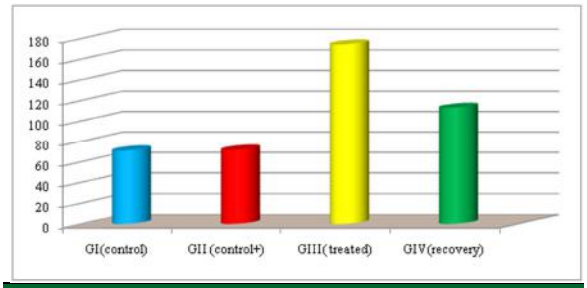


Figure 1: Mean value of Blood glucose level in all studied groups.

Table 2: Lipid peroxidation profile and defense antioxidant system response in all studied groups.

Parameters	GI -vecrl	GII +vecrl	GIII treated group with GA3	GIV Recovery group	One Way ANOVA F	One Way ANOVA P-value
Lipid peroxidation						
MDA nmol/ml	2±0.3	1.9±0.1	4.9±0.5	3.5±0.2	310.128	<0.001
Antioxidant defense						
GSH u/ml	187.9±2	192.1±65	140.3±6.8	162.1±0.8	8.194	<0.001
SOD u/ml	454±90	465.6±85	234±45.6	309.7±51	43.07	<0.001
CAT u/ml	194±7	180±11.5	90±8.4	120.4±19	236.658	<0.001

Post Hoc Analysis (LSD test)

Parameters	GI vs GII	GI vs GIII	GI vs GIV	GII vs GIII	GII vs GIV	GIII vs GIV
MDA nmol/ml	0.231	<0.001	<0.001	<0.001	<0.001	<0.001
GSH u/ml	0.804	<0.001	<0.001	0.005	0.085	<0.001
SOD u/ml	0.719	<0.001	<0.001	<0.001	<0.001	0.001
CAT u/ml	0.001	<0.001	<0.001	<0.001	<0.001	<0.001

By doing post hoc analysis, it appeared that both positive and negative control groups not differ significantly from each other in the levels of peroxidation production and antioxidant enzymes while, both control groups differ highly significantly from the treated group by GA3 indicating its pancreatic toxicity.

In the same way, control groups differ highly

significantly from recovery group, denoting that the insult became chronic.

The high significant (P<0.001) values between the treated and the recovery according to levels of MDA and anti-enzymes, indicating some improvement occurred by stoppage of the GA3[Table 2] & [Figure 2].

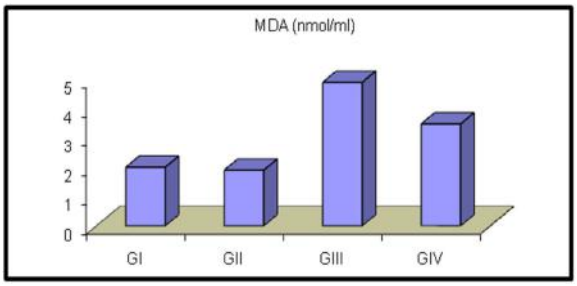


Figure 2: Lipid peroxidation profile system response in all studied groups.

There were highly significant elevation in the used diagnostic pancreatic markers (amylase and lipase enzymes) along with concomitant increase in (MDA) (marker of lipid peroxidation) and the dramatic decrease in antioxidant enzymes in the blood of the treated animals.The insult which denoted from the increase in the exocrine pancreatic enzymes didn't resolve by the recovery period from the GA3 which may suspect the occurrence of chronic pancreatitis [Table 3]& [Figure 4, 5].

By doing post hoc analysis for (P) values for amylase and lipase levels differences between the groups of the animals, the same results obtained as in [Table 2].

II- Histological results:

The histopathological examination of serial transverse sections of the pancreases of the control negative and positive groups (group I and II) were similar, so both were pooled together.

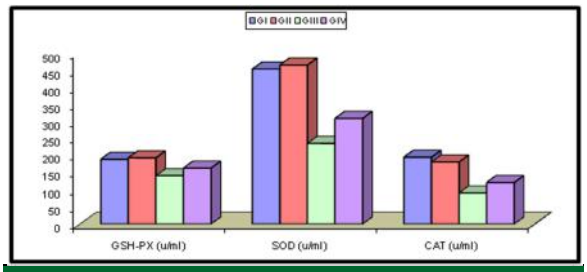


Figure 3: Antioxidant defense system response in all studied groups.

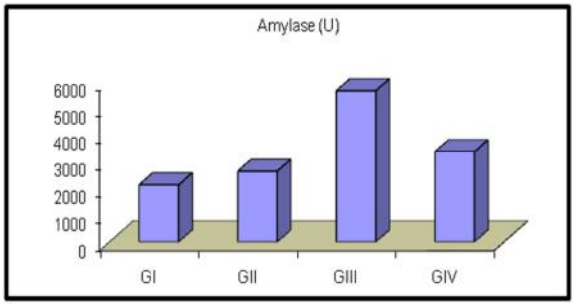


Figure 4: Mean amylase serum level in all studied groups.

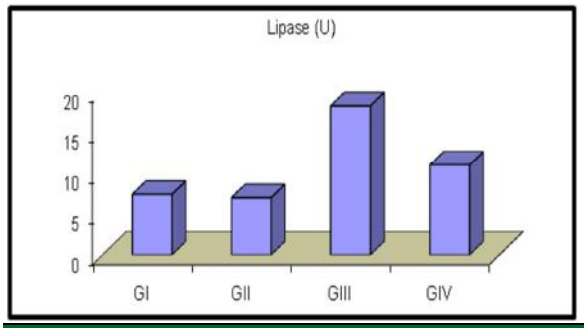


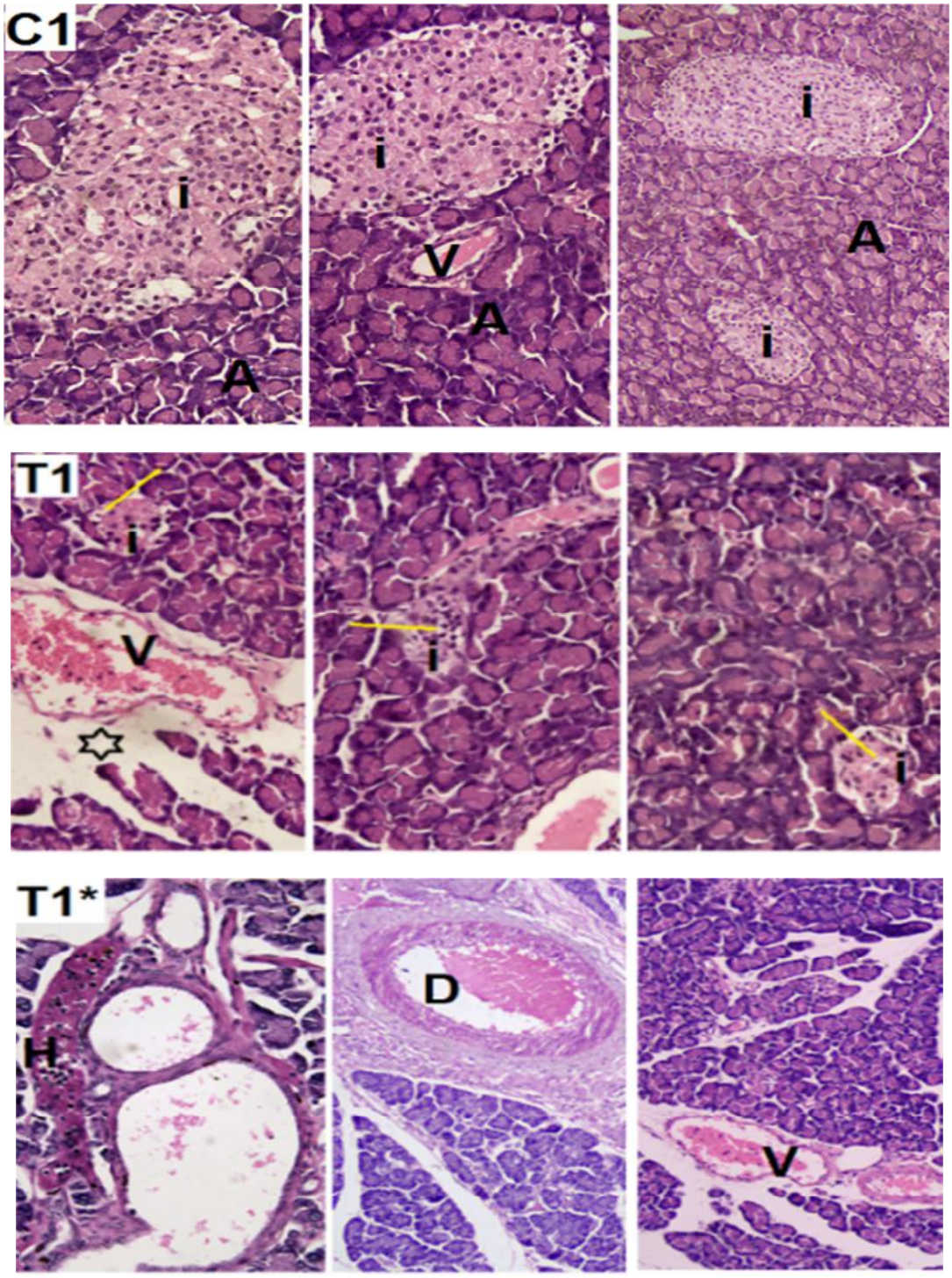
Figure5: Mean lipase serum level in all studied groups.

Table 3: Mean amylase and lipase serum level in all studied groups.

Parameters	GI	GII	GIII	GIV	One Way ANOVA	
	-ve control	+ve control	treated group	Recovering group	F	P-value
Amylase (U)	2123.48±155	2630.7±340	5672±123.5	3381.44±708.20	**68.066**	<0.001
Lipase (U)	7.35±0.34	6.94±0.81	18.24±0.7	10.963±0.27	231.42	<0.001

Post Hoc Analysis (LSD test)						
Parameters	**GI vs GII**	**GI vs GIII**	**GI vs GIV**	**GII vs GIII**	**GII vs GIV**	**GIII vs GIV**
Amylase (U)	<0.001	<0.001	<0.001	<0.001	0.001	<0.001
Lipase (U)	0.081	<0.001	<0.001	<0.001	<0.001	<0.001

The pancreas consisted of many uniformly compact arranged lobules separated from each other by scanty interlobular connective tissues. Each lobule contained two types of parenchymal tissues, the islets of Langerhans which was lightly staining clusters of cells and darker staining cells which were the acini [Figure 6, c1]. The islets of Langerhan`s separated from the surrounding pancreatic acini by a delicate reticular fibers and arranged into anastomosing cords that were vascularized by fenestrated capillaries. The most prominent endocrinal cells of the islets of Langerhan`s were alpha and beta cells [Figure 7, c2].



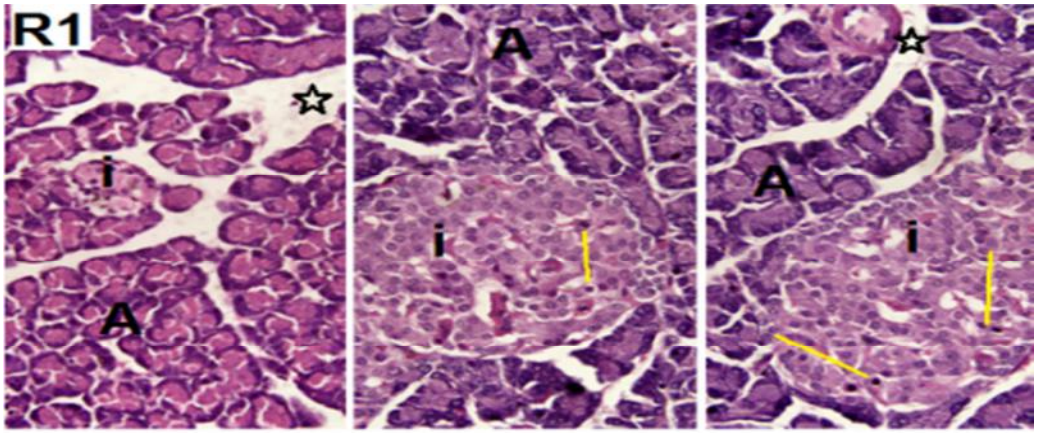
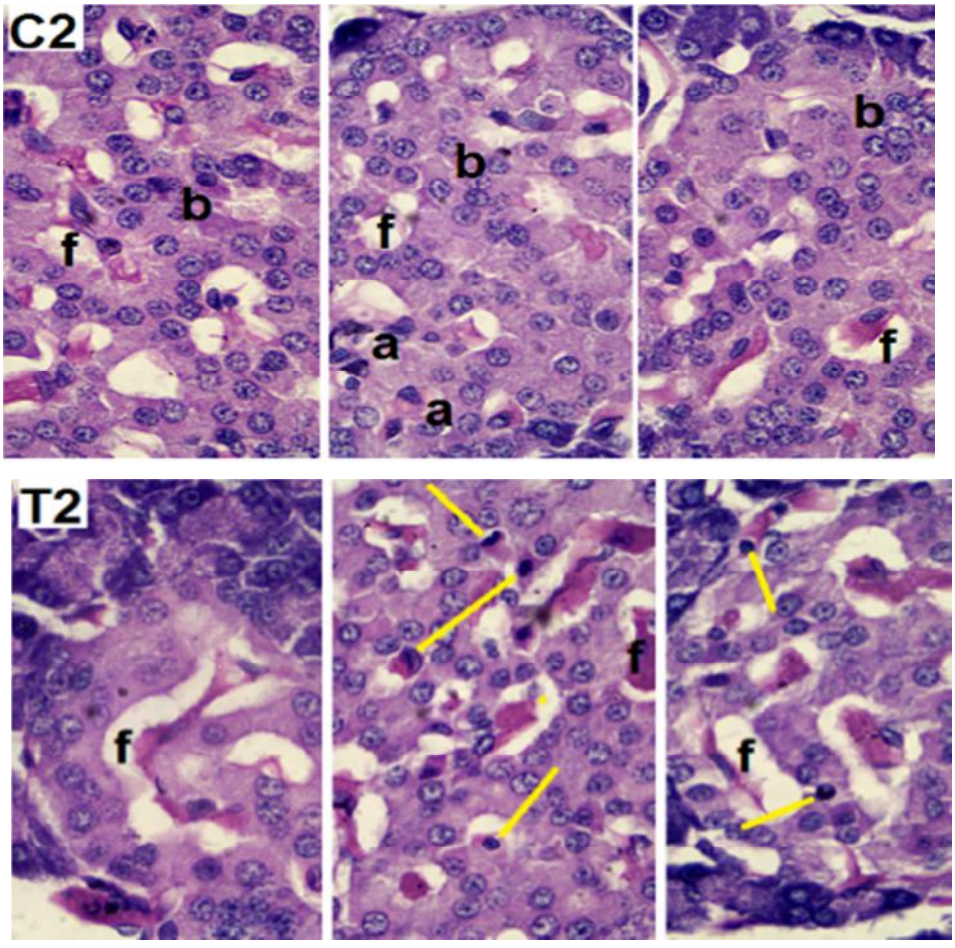


Figure 6: Photomicrographs of transverse sections of the pancreases of all studied groups demonstrating that:
(C1, Controls): the pancreas contains both types of parenchymal tissues, islets of Langerhans (i) which is lightly staining clusters of cells and the acini cells (A) which are rounded or oval contain basal basophilic nuclei and apical acidophilic cytoplasm. Notice, presence of small blood vessel (V).
(T1, treated group): showing a shrunken small islet of Langerhans (i), some cells containing pyknotic nuclei and vacuolated cytoplasm (yellow stick). Notice the presence of large dilated blood vessel (V) and wide spaces between the acini (*).
(T1*, treated group): showing also the presence of wide spaces in between the acini (*) with presence of large thick wall dilated blood vessel (V) .Notice the presence of large thick wall pancreatic duct(D).
(R1, recovery group): few cells of islets of Langerhans (i) containingpyknotic nuclei (yellow stick). The architectures of the pancreas appear nearly normal and most of the lobules had normal acinar cells (A). Notice the presence of narrow spaces between the acini (*). (Hx. & E.; X400).



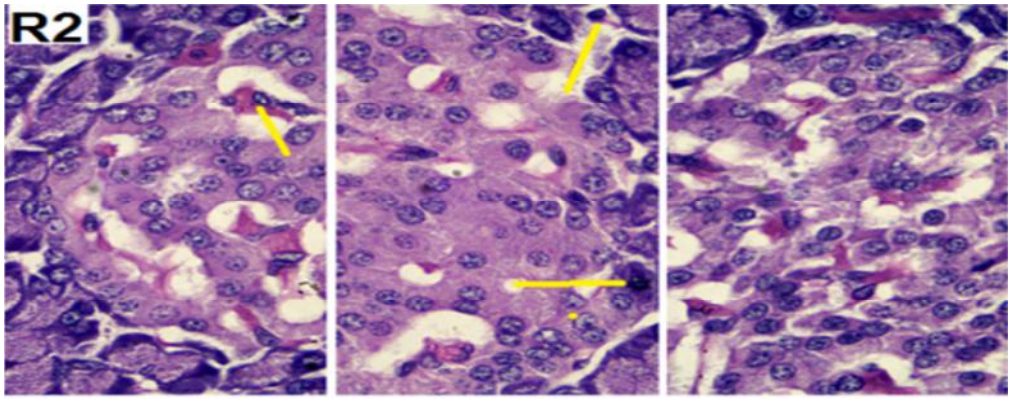
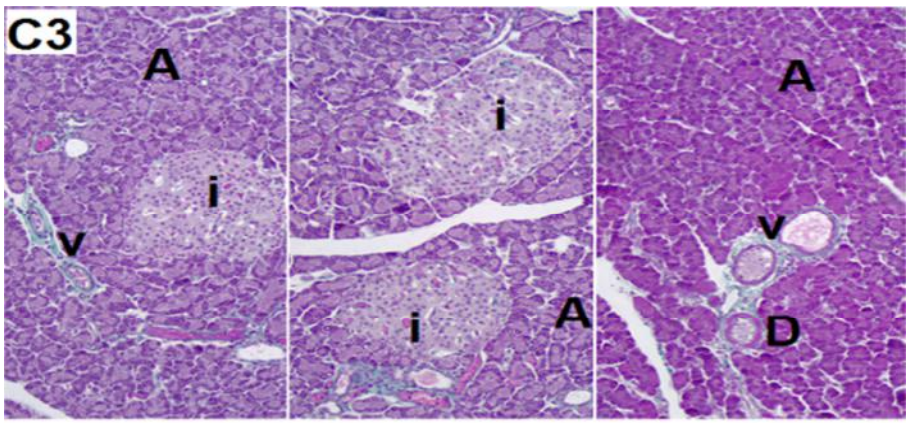


Figure 7: Photomicrographs of higher magnification of the previous figures showing that:
(C2; Controls): large islet of Langerhans containing endocrine cells beta cells (b) which have rounded vesicular nuclei with pale acidophilic cytoplasm occupying central position and alpha cells (a) which appear oval or elongated cell represented in the periphery. Note the presence of fenestrated small blood capillaries (f).
(T2; treated group): the islet of Langerhans has less population of cells and most of them has vacuolated cytoplasm and deeply stained or pyknotic nuclei (yellow stick) with presence of dilatation of the fenestrated capillary (f).
(R2, recovery group): most of the cells of the islet of Langerhans have normal appearance except few of them still have pyknotic nuclei (yellow stick)(Hx. & E.; X1000).

The beta cells were the most popular cell and occupied the central position in the islets, it had large rounded nucleus and pale cytoplasm. The alpha cells are small dark cells with small-elongated nuclei they present in the periphery [Figure 6, c1] [Figure 7, c2]. The acinar cells were pyramidal in shape containing basophilic rounded basal nuclei and apical acidophilic cytoplasm. [Figure 6, c1].Normal distribution of delicate collagen fibers were seen around islands of Langerhans, pancreatic acini and pancreatic ducts as well as in the wall of the blood vessels [Figure 8, c3]. In the same instance, In GA3treated group **(group III)** small islet of Langerhans appeared shrunken, contained less population of cells and most of them has vacuolated cytoplasm and deeply stained or pyknotic nuclei with presence of dilatation of the fenestrated capillary moreover there were large dilated blood vessel and wide spaces in between the cells.
Also, many acinar cells were irregularly arranged with presence of wide spaces in between. Some acinar nuclei were small darkly stained with acidophilic cytoplasm. The interlobular duct appeared dilated with thick wall. There were multiple congested blood capillary and extra vasated blood were seen when compared to the control groups [Figure 6, T1, T1*]. In addition, dense collagen fibers depositions were seen between acini and in the wall of the congested blood vessels [Figure 8, T3].
In contrast, the pancreases of the group IV **(recovery group)** showed many signs of improvement. Islets of Langerhans attained large size with increase beta cell populations in addition to apparently normal acinar cells which appeared normal in shape with narrow intercellular spaces when compared to GA3 treated group [Figure 6, R1] [Figure 7, R2]. In another hand, less collagen fibers deposition could see between the acini and in the wall of the congested blood vessels when compare to the treated group [Figure 8, R3].



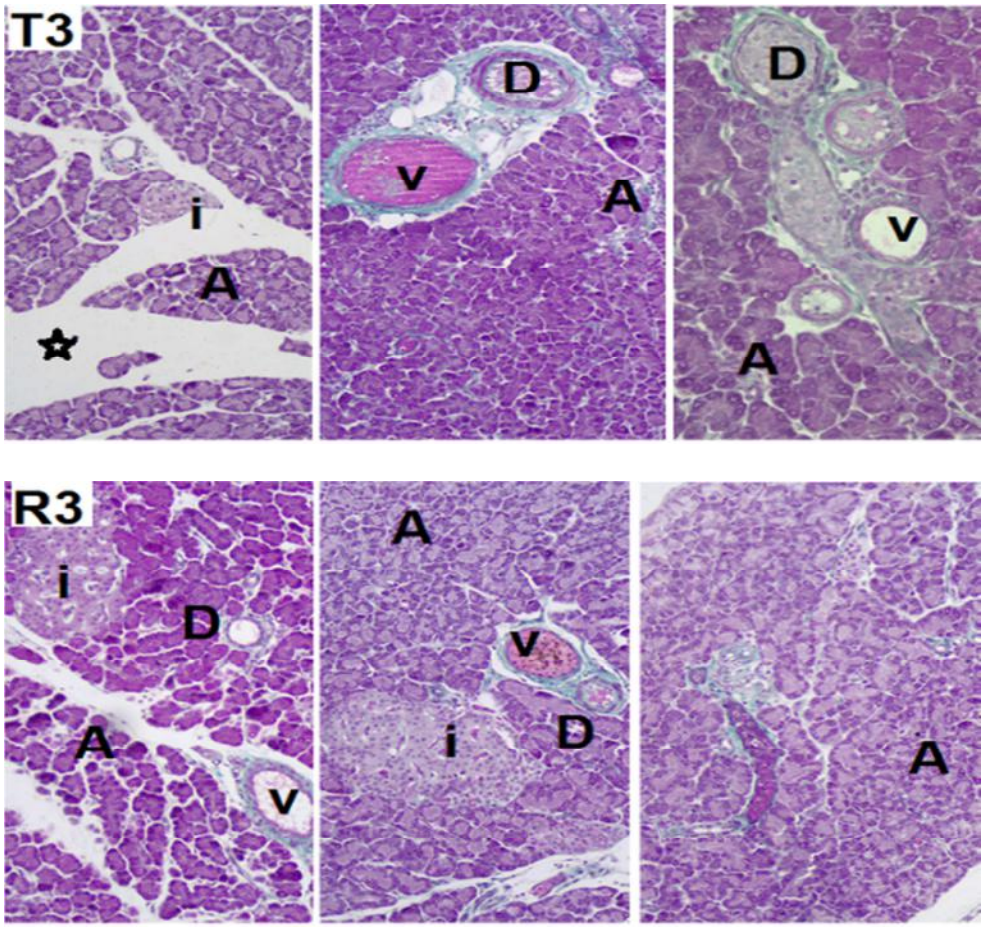
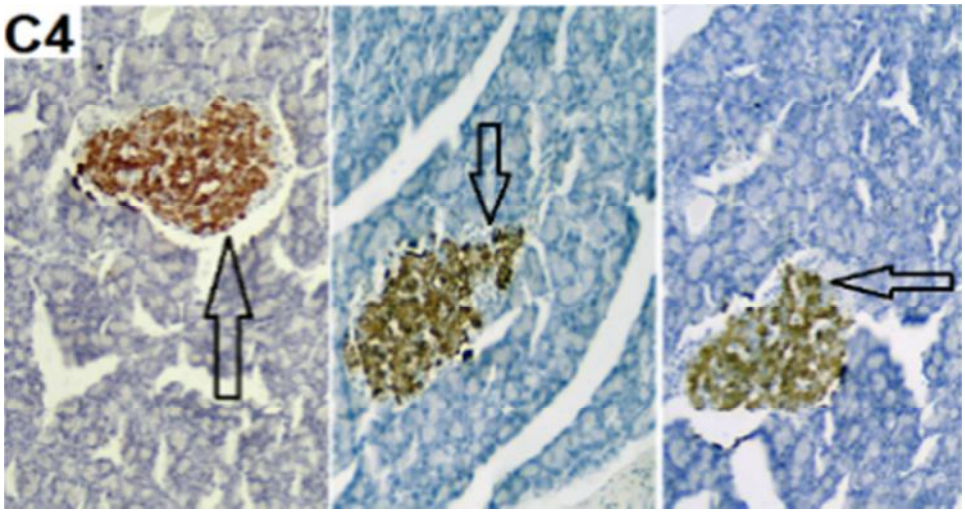


Figure 8: Photomicrographs of transverse sections of the pancreases of all studied groups demonstrating that:
C3; Controls): normal distribution of delicate collagen fibers around islands of Langerhans (i), between the pancreatic acini (A), pancreatic duct (D) and in the wall of the blood vessels (V).
(T3; treated group): dense collagen fibers deposition are seen around islands of Langerhans (i), pancreatic acini(A), pancreatic duct (D)and in the wall of the blood vessels (V).
(R3, recovery group): less deposition of the collagen fibers around islands of Langerhans (i), pancreatic acini(A), pancreatic duct (D) and in the wall of the blood vessels (v).(Masson trichrome; X 400).



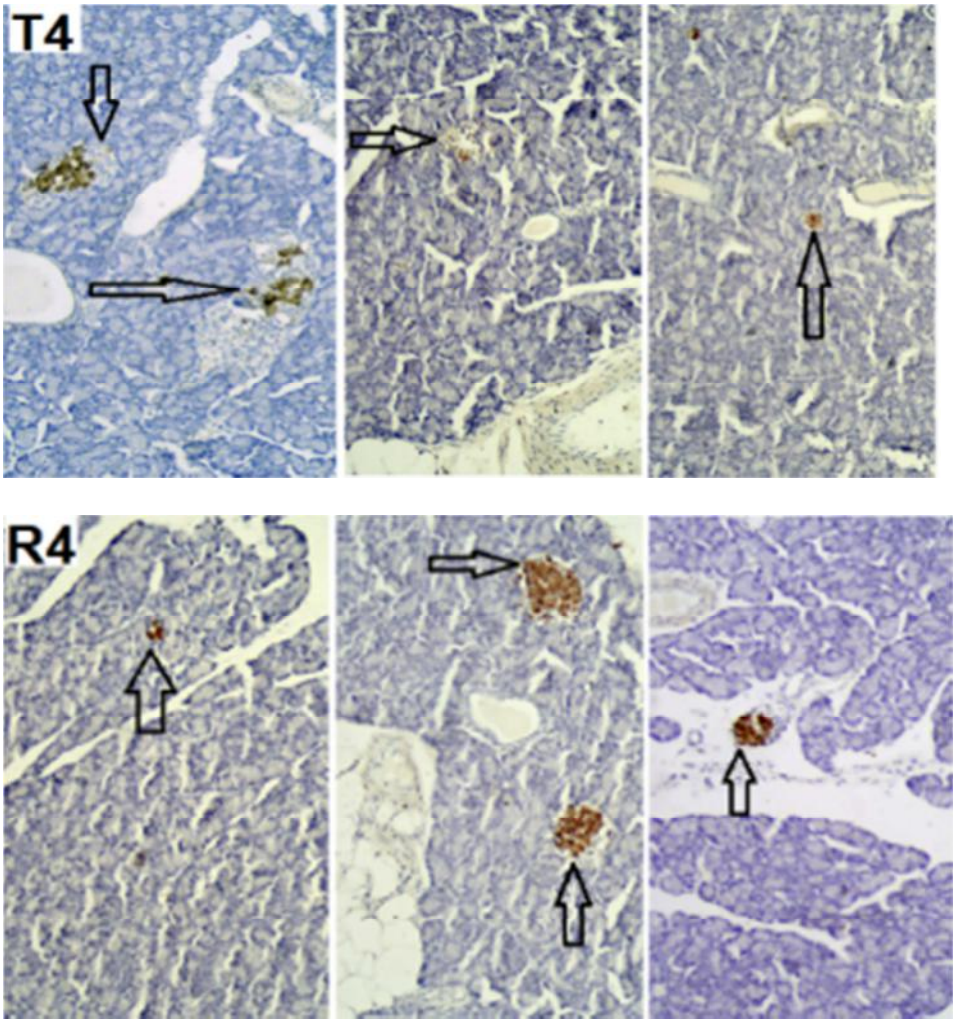


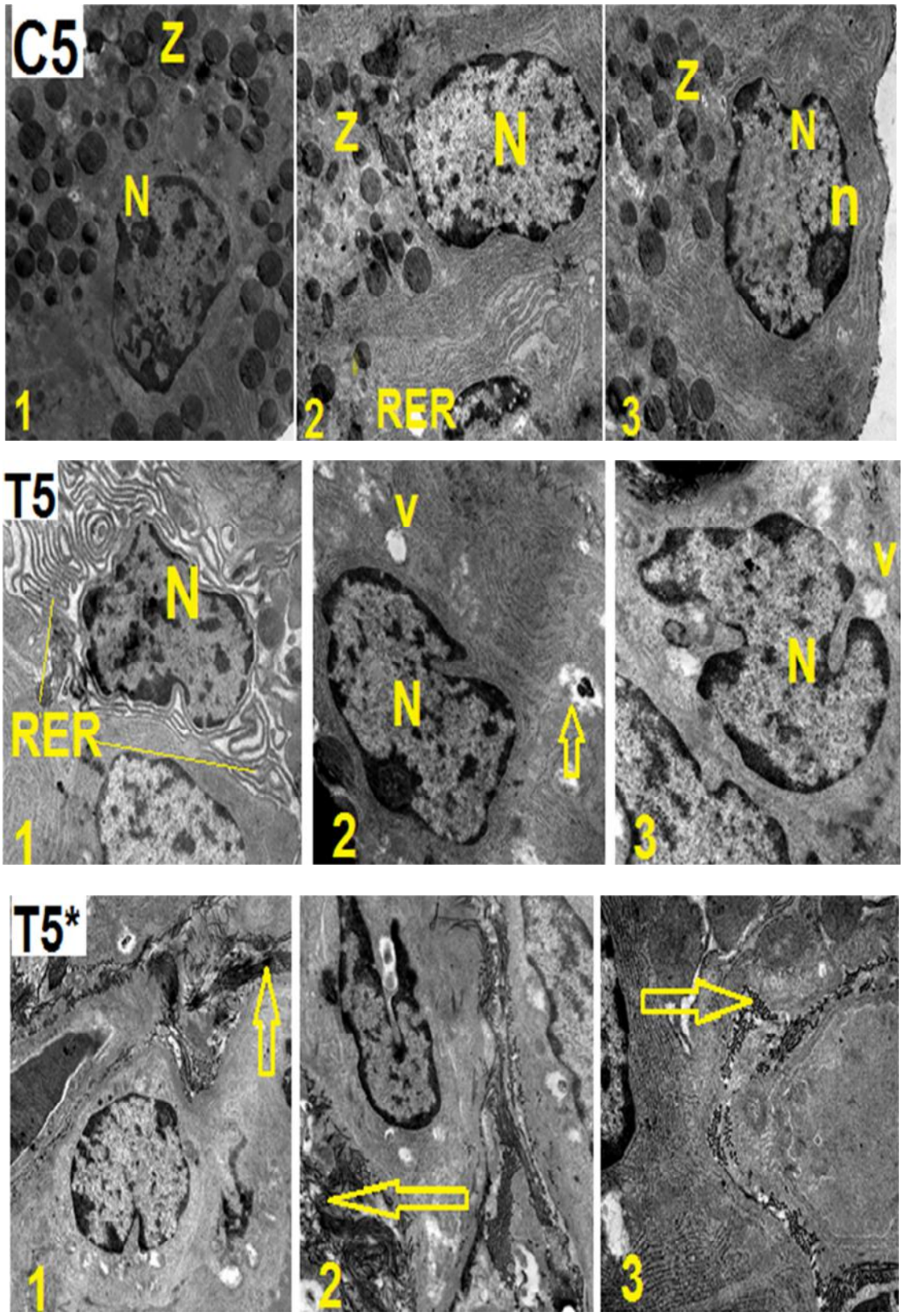
Figure 9: Photomicrographs of transverse sections of insulin immunohistochemical staining of pancreases of all studied groups demonstrating that:
(C4; Control): showing that the pancreatic b-cells has positive insulin immunostaining (arrows) distributed over the center of pancreatic islets and stained with deep brown color.
(T4; treated group): showing marked decrease in the size of islands of Langerhans with negative immuno-staining particles for insulin (arrows).
(R4; recovery group): partial increase in the size and staining particles of the islands of Langerhans (arrows). (Immuno-staining for anti-insulin antibodies X400.).

Immunohistochemical Findings

Immunohistochemical staining of the pancreas of control groups demonstrated a strong positive insulin-immuno-reactive brown spots in the beta cells of the islets of Langerhans [Figure 9, C4]. In contrast in-group III the islets were small, atrophied and insulin-immuno-reactive spots were scanty or almost lost [Figure 9, T4]**,** but some improvements were seen in the islets' structure of the recovery group whereas more insulin-immuno-reactivity cells appeared positive [Figure 9, R4].

Electron microscopic examination of the acinar cells:

Ultra structural finding of the acinar cells in the control groups showed normal euchromatic nucleus containing prominent nucleolus. The cytoplasm contained characteristic multiple rounded electron dense zymogen granules in the apical part, regularly arranged rough endoplasmic reticulum, normal mitochondria and lysosomes [Figure 10, C5].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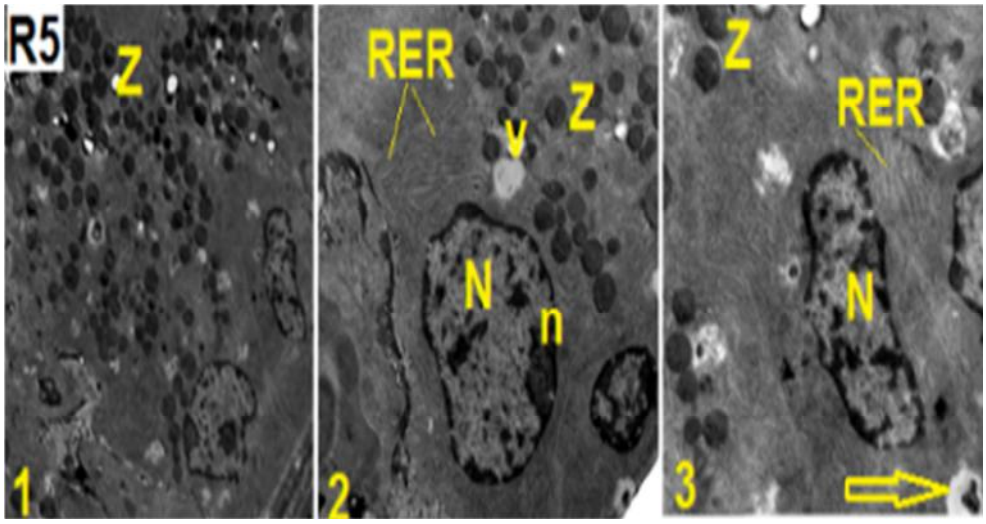

Figure 10: Electron micrographs of the pancreatic acinar cells of all studied groups demonstrating that:
(C5; Controls): the acinar cells has euchromatic nuclei (N), sharply demarcated and prominent nucleoli (n). Well-developed cisternae of regular rough endoplasmic reticulum (RER), and numerous electron dense secretory zymogenic granules (Z) of variable sizes in the apical part. (TEM; 1, 2&3 x 12000)
(T5; treated group): showing marked changes in pancreatic acini represented by irregular contours of nuclei (N), irregular dilated rough endoplasmic reticulum (RER), absences of secretory granules with presence of many cytoplasmic vacuolations (V) and presence of auotophagic vacuole (arrow). (TEM; 1x 12000 - 2x 15000- 3x 20000)
(T5*; treated group): showing marked deposition of collagenous fibers in different directions around the acinar cells (yellow arrow). (TEM; 1x 10000 - 2x 15000 - 3x 20000)
(R5; recovery group): showing partial improvement represented by regular contours of the nuclei (N) ,appearance of some zymogen granules (Z),the regular rough endoplasmic (RER) returned its regulatory and flatness. Notice that few cytoplasmic vacuoles and phagosomes still present. (TEM; 1x 6000 - 2x 12000- 3x 15000).

In GA3 treated group (group III) prominent degenerative changes in pancreatic acini were seen in the form of irregular contours of nuclei, dilated irregular rough endoplasmic reticulum**,** the quantity of the secretory granules were markedly decrease with presence of multiple cytoplasmic vacuolations in addition to presence of auotophagic vacuole.[Figure 10, T5]. In addition, there were dense collagen fibers deposition in different direction around the acinar cells [Figure 10, T5*].
In recovery group partial improvement were notable by regular contours of nuclei and increase in the population of zymogen granules, more over the rough endoplasmic reticulum return its flattened regular shape. However few acinar cells still had few vacuoles [Figure 10, R5].

III- Morphometric results:
The morphometric results represent significant reduction($P < 0.001$) in the number of islets/pancreas sections and the number of beta cells/islet in treated group III in comparison to that of control groups. However, these parameters, in recovery group, were increase in the quantity but still significantly decreased when compared with the control [Figure 11][Table 4].

Table 4: Number of islets/pancreatic section (N/10 mm^2) and number of beta cells/islet (N/1000um^2).

Groups	Number of islets/pancreatic section (N/10 mm^2)	Number of beta cells/islet (N/1000um^2)
GI (control)	17.89 ± 0.531	9.89 ± 0.481
GII (control+)	17.25 ± 0.481	9.18 ± 0.357
GIII (treated)	3.91 ± 0.240*	1.99 ± 0.164*
GIV(recovery)	8.21 ± 0.341**	4.64±0.143**

Values are expressed as mean ± SEM.
*Test of significance between GI (control) and GIII (treated) rats at $p < 0.001$.
**Test of significance between GIV (recovery) and GI (control) rats at $p < 0.001$

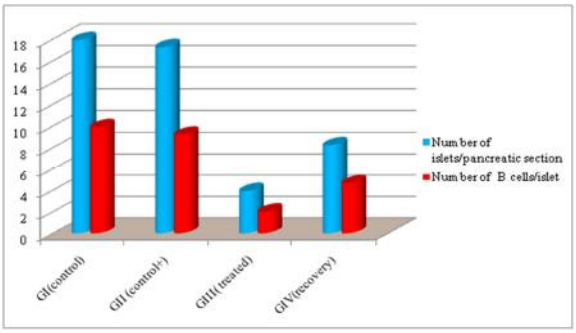

Figure 11: Mean value of number of islets/pancreas and number of beta cells/islet in all studied groups

DISCUSSION

There has been an increase in the incidence of acute pancreatitis reported worldwide. Despite improvements in access to care, imaging and interventional techniques, acute pancreatitis continues to be associated with significant morbidity and mortality.[23]In the case of experimental animals, especially rodents, numerous chemical toxicants have been identified for the exocrine pancreas and, to a lesser degree, the endocrine component.[41]GA3 is used to increase fruit size, increase cluster size (in grapes), delay ripening of citrus fruits, and speed up flowering of strawberries.[10,43]

In the present study, in group III, biochemical, microscopical and ultra-structural findings provided that GA3 expose the pancreases to oxidative stress. Whereas, there were highly significant increase of the mean serum amylase and lipase levels indicating presence of oxidative stress which led to pancreatitis. Cruz-Santamaria.et al., (2012)[13]stated that pathogenesis of acute pancreatitis is based on the intracellular autodigestive process, which triggers local and systemic inflammatory response via release of mediators in parenchyma. These biochemical interactions eventually lead to acinar injury, interstitial edema and tissue compromise. Recently, Mirmalek et al., (2016)[29]confirm the present results, they stated that serum lipase and amylase activities, most commonly used biomarkers in AP.

In the same way, the histological finding in the treated group of the present study, there were wide intercellular spaces between the islets and acinar cells which indicate presence of interstitial edema, in addition to presence of many signs of degeneration in the form of presence of many cells with dark small sized pyknotic nuclei and vacuolated cytoplasm in both islets and aciniar cells. Moreover an increase of fibrous tissue deposition were seen. These finding provide exposure of the pancreatic tissue to inflammatory reaction .Similar suggestion was observed by Parimal et al.,(2000)[35] who cited that an increased number of cells with pyknotic nuclei, vacuolations and extensive edematous swelling in many acinar cells as well as appearance of cytoplasmic vacuoles as an indicative criterion for early pancreatitis.

In the group III of the present study the major lipid peroxidation product MDA increased significantly in comparison with the control groups. Also, GH-Px and antioxidant enzymes decreased significantly indicating highly significant decrease in scavenging power of the tissue due to the toxic effect of GA3.Similar suggestion was cited by Abu-Hilal, (2006)[4]who reported that oxidative stress is one of the pivotal mechanisms of AP. Excessive reactive oxygen species (ROS) provoke inflammation and development of pancreatitis through zymogen de-granulation, granulocyte migration, tissue necrosis, and increased amylase and lipase activity. In fact, ROS as products of oxidation and peroxidation metabolism, are instantly detoxified by natural scavengers and antioxidants agents under normal conditions.[9] In AP, overproduction of ROS and the impaired neutralization ability of scavengers results in ROS accumulation in pancreatic tissue.[8]

In the current study, in a group III, presence of high blood glucose level and negative insulin immuno-reactivity with reduction of number of beta cells, indicated that GA3 cause considerable distraction of beta cell, which led toincrease generation of ROS in the pancreas which leading to oxidative stress and the end result is diabetes mallets. The latter itself expose the pancreatic tissue to oxidative stress. Kanter, et al., (2004)[24] and Lai, (2008)[26] confirm the present suggestion, and they reported that diabetes has been shown to be a state of free radicals overproduction resulting from hyperglycemia. Factors that attributed to the formation of free radicals may include not only elevated non-enzymatic and auto oxidative glycosylation, but also metabolic stress as a result of alterations in energy metabolism, inflammatory mediator's levels and the status of antioxidant defense. Hamdena, et al., (2011)[21] cited thatdecrease in both antioxidant capacity and insulin activity/sensitivity led to commonly observe diabetic complications. Also, Tuluce and Celik,(2006)[45]reported that antioxidant

enzyme activities were significantly decreased in the erythrocyte, liver, and brain tissues of rats treated with GA3**.** In additionPadgett et al., (2013)[34] observed that both apoptosis and necrosis have been reported to be responsible for cell death in pancreatic β-cells exposed to immune and inflammatory cytokines.

In the present study, the destruction of β-cell and reduction in the islet cell mass in the treated rats' pancreas confirm the cytotoxic activity of GA3. Similarly Evans et al., (2003)[16] and Robertson, (2006)[37]provided that oxidative stress is associated with the molecular mechanism of the decreased insulin biosynthesis and secretion, which is the main etiology of glucose toxicity. Indeed, it was suggested that the pancreas may be more susceptible to oxidative stress than other tissues and organs, because pancreatic islet cells show extremely weak manifestation of anti-oxidative enzymes. The authorsadded that oxidative stress plays a key role in causing insulin resistance and beta cell dysfunction by their ability to activate stress-sensitive signaling pathways.

In the present study, in-group III, prominent increase of collagenous fiber deposition in between the pancreatic lobules and in the wall of its duct and blood vessels were cited by light and electron microscopy, the attributed causes were referred also to the proved oxidative stress. Similarly Zhang et al., (2008)[48] and Yan et al., (2012)[47]reported that an increase in collagen synthesis, during inflammation of pancreas, resulted from activation of pancreatic stellate cells in response to lipid peroxidation.

Ultra structural fining of the acinar cells clarified the degenerative effect of the pancreatic acinar cells of GA3 treated rats ,in the form of dilatation of rough endoplasmic reticulum, cytoplasmic vacuoles, and decrease of zymogen granules in its cytoplasm. These results were in accordance with Ozcan et al., (2004)[33] who reported that dilatation of rough endoplasmic reticulum indicate increased endoplasmic reticulum stress. Also, Adeghate et al., (2006)[5] stated that endoplasmic reticulum expansion may be the early sign of cell damage in addition to intracellular vacuoles as a result of lipid accumulation as a consequence of enhanced lipolysis and adipocyte dysfunction causes impairment of oxidation in the mitochondria.[30]

By stoppage of GA3 for 6weeks, the blood glucose level, lipid peroxidation profile and levels of amylase and lipase didn't improve completely and still increased above control levels. The elevation which followed by decrease on cessation of GA3 is the corner stone in the diagnosis of AP. Also, many signs of improvement were seen at the tissue level after cessation of GA3, as that the islets of Langerhans became larger in size with increase beta cell population in addition to apparently normal acinar cells with narrow intercellular spaces when compared with GA3 treated group. In another hand, less collagen fibers deposition could see between the acini and in the wall of the congested blood vessels indicating that the pancreatic tissue could eliminate the oxidative stress but within limit. Similarly Rong and Distelhorst,(2008)[39]reported improvement of histopathological criteria of the renal cortex of GA3 recovery group ,the authors observed self-regeneration occur with increased antioxidant enzymes and decreased MDA in kidney in comparison with GA3 treated group but oxidative stress still present.

Insulin-immunoreactivity and morphometric results of the recovery group in the current study confirmed evidence of improvements in the islets' structure whereas more beta cells appeared positive.Abd El Maksoud et al.(1996)[1] who stated that following GA3 withdrawal, a few hepatic cells became nearly similar to those of the control group, while the majority of cells remained affected. Also**;** Saly, (1998)[40] noticed partial improvement of the destructive chronic toxic effects of GA3 on liver, heart and kidney after four weeks of recovery.

In our study , notable ultra-structures improvement in the acinar cells were seen as regular contours of nuclei and increase in the population of zymogen granules, more over the rough endoplasmic reticulum return its regular flattened shape. However, few acinar cells still had few cytoplasmic vacuoles. The above mentioned results coincided withAbou-zeid and Abd-Ellah, (2015)[3]who reported that recovery of some Purkinje neuron was observed where ultra-structure alterations were less evident than those in the treated animals. Their cytoplasm showed that most of RER appeared in nearly restored condition.

In contrast, persistence of presence of phagosomes in the acinar cells inthe recovery group in spite of presence of many signs of improvement indicate continues trials of the defense system to auto regulate and enhance apoptosisto eliminate the affected died cells. In agreement with this suggestion, Rong and Distelhorst, (2008)[39]reported that the apoptosis associated with the recovery phase has been contributed to the remodeling of injured tubules and to facilitate their return to a normal structural and functional state.

CONCLUSION

Ingestion of residual amount of GA3 which present insome fruits and vegetables resulted indisturbed structure and functions of the pancreas as a result of exposure to oxidative stress and 6 weeks is not enough to complete full recovery.

Recommendation:

1-More studies are needed on the mechanism of effect of residue of GA3 on the human being and explore a suitable antioxidant, which can overcome the negative impact.

2- Avoid eating the fruits in the off-season specially which attained large size furthermore, wash and macerate fruits before eating to get rid of the residue of GA3.

3- Educate peasants about the health hazers caused by abusing GA3 on them and on the consuming persons, in addition to application of rules and regulations for the ideal use of GA3 and punish the outlaws.

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