Original Article

The Protective Impact of Lycopene and Folic Acid Supplementation Against Nicotine Toxicity on Pancreatic Islets in Adult Male Albino Rats: Biochemical and Immuno-Histochemical Study

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ABSTRACT

Introduction: It is reported that dysfunctioning of islets and elevated levels of fasting blood glucose are found in rats exposed to nicotine. Lycopene is thought to have a potential role as an effective antioxidant in the prevention of chronic diseases associated with oxidative stress. Folic acid is a water-soluble vitamin B that is essential for amino acid metabolism.

Aim of the Study: To explore the effects of nicotine toxicity on pancreatic islets and protective impact of lycopene & folic acid supplementation.

Material and Methods: Fifty healthy adult male albino rats were separated into five groups. Control , Nicotine treated at which, rats were injected intraperitoneal by nicotine 3mg/kg daily for a period of 3 weeks . Nicotine + Lycopene treated group at which rats received lycopene at a dosage of 10 mg/kg b.wt. daily in combination with nicotine treatment for 3 weeks. Nicotine + Folic acid treated group at which ,rats were injected nicotine and given Folic acid orally at a dosage of 36 µg/kg. b.wt and recovery group at which rats kept for one month after 3 weeks of nicotine injection. then pancreatic tissues were examined for histopathological and immunohistochemical changes.

Results: Nicotine treated group showed degenerated pancreatic islets with ill-defined outline . Numerous collagen fibers were present within and around the pancreatic islets in masson stained pancreatic sections, Strong INOs immunoreactivity but anti-insulin immuno expression has decreased. Lycopene and Folic acid reduces toxic effect of nicotine on pancreatic islets, but Folic acid revealed a significant decrease in collagen fibers, INOs immunoreactivity and significant increase in anti-insulin immuno expression compared with that in Lycopene group.

Conclusion: Use of lycopene during the period of nicotine injection considered to have a protecting influence on pancreatic islets. Meanwhile The use of Folic acid has a more protection than Lycopene.

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Key Words: Folic acid and pancreatic islets, lycopene, nicotine.

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INTRODUCTION

Nicotine which is a main constituent of tobacco and cigarette smoking, considered as an addictive factor and has been categorized as a medication of abuse.[1] It leads to oxidative destruction in the tissues and nucleic acids causing many diseases.[2] Also nicotine exposure has been stated to interrupt the defense mechanisms of endogenous antioxidant by decreasing catalase and SOD[3] It is noteworthy that islet dysfunction and elevated fasting blood glucose levels were found in rats when treated with nicotine.[4] Nicotine exposure provokes the production of Reactive oxygen species (R0S) in the body.[5] Alternatively, (ROS) increases both types of diabetes. Based on that, causes of oxidative stress in diabetes may comprise the auto-oxidation of glucose.[6]

Lycopene (LYC) is considered as a main carotenoid, obtained mainly from tomato, which is an essential part of the ordinary Egyptian food. Furthermore, it is proved to have antiapoptotic properties.[7] It has several biochemical functions like an antioxidant scavenger, anti hyperlipidemic mediator and act as an inhibitor of prothrombotic and proinflammatory factors.[8] Supplementation of LYC has been verified for its protective outcome against the destructive oxidative injury of tissues triggered by ecological pollutants.[9] It is thought that lycopene has a probable role in the inhibition of chronic diseases related to oxidative stress.[10] The antioxidant action of lycopene is mostly reliant on its O2 and -OH hunting actvitiy.[11] So administration of lycopene decrease generation of ROS and increase synthesis of GSH which is the main antioxidant in cells, GSH is usually exhausted in case of acute pancreatitis, and this is believed to increase intensity of the disease.[12]

Also lycopene is effective in lipid per0xidation inhibition and destruction of DNA by widespread reactive 0xygen species[13] The open β-cycle in its structure is responsible for its high antioxidant capacity[14] adequate folate intake is a critical factor in reducing the incidence of neural tube defects and has been implicated in numerous other adverse health conditions essential micronutrient that is vital for normal cellular function; adequate folate intake is a critical factor in reducing the incidence of neural tube

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defects and has been implicated in numerous other adverse health conditions.

Folic acid (FA) is considered as a water-soluble B vitamin which is important for the metabolism of amino acid, is also called as vitamin B9[15]. It is a micronutrient which is important for regular function of the cell .So sufficient intake of the folate is considered as a critical factor in decreasing the frequency of defects occurred in neural tube and has been concerned in numerous other health conditions[16]. Folate deficiency could stimulate overproduction of ROS and increase the intracellular calcium leading to apoptosis which occur in many cell types. This leads to the pathogenesis of many diseases like neural tube defects in fetus ,cardiovascular diseases, anemia, , cancer, and Alzheimer's disease[17].

MATERIALS AND METHODS

Animals & experimental plan

This study was conducted on 50 healthy adult male albino rats (2 months-old) weighting 180-200 gram. The rats were got from the Faculty of Veterinary Medicine, Laboratory Animals Unit, Zagazig University, Egypt. The rats were kept in plastic cages to evade any metallic contact beneath environmental laboratory situation at 20 ± 2°C. The rats were exposed to an organized photo period (14 h: 10 h light:dark), water ad-libitum and a standard diet were allowed. The experimental protocol was agreed by the Ethical Committee of Benha University.

After an accommodation period of one week, rats were haphazardly distributed into 5 equal groups: I, II,III , IV& V

Group I (control group): Ten rats were divided into:

- Group Ia (negative control group) (4 rats): Each rat was given only systematic diet and tap water to measure the major standards .
- Group Ib (saline) (3 rats): Each rat received a daily (IP) injection of 1 mL 0.9% physiological saline.[4]
- Group Ic (corn oil) (3 rats): Each rat received a daily 0.5 ml of corn oil through oral gavage.[18]

Group II (nicotine treated group): ten rats that were given nicotine tartrate (dissolved in 0.9% physiological saline) through intraperitoneal injection at a dose of 3 mg/kg B.Wt daily for 3 weeks at 16:00 h every day to evade diurnal variant.[19]

Group III (nicotine + lycopene treated group); ten rats treated with nicotine intraperitoneally (3 mg/kg B.Wt) accompanied with lycopene dissolved in corn oil at a dose of 10 mg/kg bw orally through a gavage needle daily for 21 days[19]

Group IV (nicotine +folic acid treated group); ten rats that were treated with folic acid at a dose 36 µg/kg B.Wt/day for 21 days orally by a gavage needle in combination with nicotine daily for 21 days[5]

Group V (Recovery group): ten rats kept for one month after 21 days Treatment of nicotine

Reagents

- Nicotine hydrogen tartrate, purchased from Sigma-Aldrich as a white powder containing 100 mg nicotine which was dissolved in 100 ml 0.9% physiological saline so each 1 ml contain 1mg nicotine.
- Lycopene 20 mg soft gels purchased online from now foods Egypt dissolved in corn oil and given by gavage needle every day for 21 day.
- Folic acid is a product of E1-Nile Company for chemicals & pharmaceutical, Egypt, supplied as tablets (5 mg). Each tablet was dissolved in 100 ml refined water. Each mL of the formed suspension contains 50 µg of folic acid. The drug suspension was shaken to confirm uniform distribution of the drug before administration of the required dose.
- Biochemical analysis:

1. Glucose measurement: After the experimental period was ended according to timing mentioned in each group, We kept the animals fasting overnight then blood samples were collected from retro-orbital veins in the next morning. Glucose oxidase enzyme kit (E. Merck, India) was used for evaluation of blood glucose from all samples.
2. HbA1C Measurement: The collected blood samples were mixed with EDTA (ethylene diamine tetra acetic acid) and were used for evaluation of glycated haemoglobin (HbA1C) subsequent to the ion exchange resin-based method.
3. Insulin Determination: Plasma insulin was determined by (ELISA) enzyme linked immunosorbent assay using the Cayman chemicals kit, USA. The variation intra assay was 4.9%. There was no inter-assay variation because the samples were run at the same time . The insulin level in plasma was conveyed in µIU/ml.
4. Measurement of glutathione level: GSH was determined in pancreas . Homogenates of pancreas (20 µl) were mixed with 10 µl of 5,5'-dithiobis- 2-nitrobenzoic acid and 200 µl of PBS. Absorbance was taken at 412 nm After 15 min of incubation. The results were conveyed as mM/mg protein[20].
5. Measurement of superoxide dismutase (SOD) activity: The NBT method was used to measure superoxide dismutase (SOD) activity, which is dependent on inhibition of NBT reduction by SOD (21). Briefly, 2.5 mL of 0.05 mol sodium carbonate solution (pH 10) was mixed with 0.1

mL of 3 mmol/L EDTA, 3 mmol/L xanthine, 1.5 mg/mL bovine serum albumin, and 0.75 mmol/L NBT. , and mitochondrial samples. The reaction was started by adding 0.1 ml of 56 µmol/ml xanthine oxidase. After a 30 min incubation period, the reaction was terminated by mixing 6 mmol/L CuCl2 and centrifuged at 350 g for 10 min. Formazan blue absorbance was measured at 560 nm. The relative uptake was then converted to a unit of SOD activity/ mg protein, where one unit of SOD activity was equivalent to the amount of SOD that caused a 50% reduction in the base rate of NBT reduction[21].

6. Quantification of MDA was performed after the thiobarbituric acid (TBA) test. Pancreatic homogenate (2 mL) was mixed with 1 mL of 20% TCA (v/v) and 1 mL of 0.67% TBA (v/v) and boiled for 10 min. After cooling, the mixture was filtered through a Whatman filter paper and the filtrate reading was performed at 530 nm. MDA formed was quantified with TBA and used as an indicator of lipid peroxidation. The results were expressed as nano moles of MDA per mg of protein using the molar extinction coefficient (1.56 × 10 5 cm2/mmol)[22].

Histological and immunohistochemical examinations

At the end of experiment according to timing mentioned in each group, the rats have been anesthetized using an intraperitoneal sodium pentobarbital injection (Nembutal, 30 mg/kg B.Wt) for sacrificing. The pancreatic tissues have been processed for a light microscopic study. They were fixed in 10 % formaline for 1 day and were handled to make paraffin sections at 5 µm thickness.

To evaluate the degree of inflammation, the sections were stained with hematoxylin and eosin (H&E) in each group to demonstrate the general histological structure and stained with Masson's trichrome stain to detect the collagen fibers in the tissues, respectively these sections were microscopically examined for any histopathological alterations.

In immunohistochemistry ,the tissue sections were deparaffinized using xylene, immersed in 3 % hydrogen peroxide to reduce endogenous peroxidase activity and warmed in sodium citrate solution (pH= 6.9) for 15 min for antigen recovery. The tissue sections were kept in 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's hematoxylin.[23]

Detection of inducible nitric oxide synthase (iNOS) antibody as a marker of inflammation.The primary antibody used was rabbit polyclonal iNOS antibody. It was supplied at a dilution of 1µg/mL and kept with the slides for 1 hour at room temperature.[24]

All tissue specimens were inspected using the light microscopy. Image acquisition was achieved with a digital microscope camera (Leica Qwin 500, Leica, Cambridge, England) computer system.

Morphometrical study

Successive sections stained with insulin immunohistochemical staining and masson's trichrome staining were analyzed morphometrically by using 500 image analyzer computer system of Leica Qwin . The standard area was measuring 7286, 78 µm . This was done in 5 non overlying fields of 5 different sections from 5 different rats in each group at × 400. for revealing :

a. Area % of the immunopositive reaction of insulin and iNOS in islets of Langerhans in sections were stained with insulin immunostained.

b. Area percentage of collagen fibers deposition, sections stained with Masson's trichrome were used.

Statistical analysis

From all groups data were expressed as (mean ± SD). The data attained from the biochemical records and image analyzer were subjected to (SPSS program; version 20.0 for windows, SPSS Inc., Chicago,IL). Statistical analysis using (One Way ANOVA) with LSD . The results were considered to be significant when the (P) value was≤0.05.

RESULTS

Biochemical results

Mean GSH, SOD & Insulin levels were reduced significantly in nicotine treated group in comparing with control group ($P \leq 0.05$), while in nicotine & Folic acid treated group (group IV) these levels were increased significantly in comparing with nicotine treated and recovery groups (groups II & V) ($P \leq 0.05$) and insignificantly decreased in comparing with control group ($P > 0.05$), In nicotine & lycopene treated group (group III) levels of GSH & Insulin were significantly reduced in comparing with control group ($P \leq 0.05$) and insignificantly decreased in comparing with nicotine & folic acid treated group (group IV) ($P > 0.05$) but they were increased significantly in comparing with nicotine treated and recovery groups (groups II & V) ($P \leq 0.05$). levels of SOD in nicotine & lycopene treated group (group III) were insignificantly decreased in comparing with control and nicotine & Folic acid treated groups ($P > 0.05$) but they were significantly increased in comparing with nicotine treated and recovery groups (groups II & V) ($P \leq 0.05$) (Tables 1,3,6, Histograms 1,3,6).

Mean MDA, FBG & HbA1c levels were increased significantly in nicotine treated group compared to control group ($P \leq 0.05$), while in nicotine & Folic acid treated group (group IV) these levels were decreased significantly in comparing with nicotine treated and recovery groups (groups II & V) ($P \leq 0.05$) and increased

insignificantly when compared to control group ($P > 0.05$), in nicotine & lycopene treated group (group III) these levels were insignificantly increased in comparing with control and nicotine & Folic acid treated groups ($P > 0.05$) but they were significantly decreased in comparing with nicotine treated and recovery groups (groups II & V) ($P \leq 0.05$) (Tables 2,4,5, Histograms 2,4,5).

Microscopic Results

Histological stains

H &E stain

Group I (Control group)

Tissue analysis of control subclasses; Ia, Ib and Ic revealed nearly the same configuration. We used figures of the control subgroup Ic to discriminate with other groups. Tissue sections of the pancreatic islets of the control group (GI) revealed centrally placed beta cells with rounded and pale nuclei and peripherally placed alpha cells with oval darkly stained nuclei, the islets appears as threads of cells separated by blood capillaries .The islet cells have lighter stained cytoplasm than the surrounding exocrine cells (Figure 1A).

Group II (Nicotine treated group)

Pancreas in nicotine treated group exhibited islets with ill-defined outline. There is decreased number of cells of islets. There is Empty spaces between cords of islets and small foci of hemorrhage in between some degenerated cells. Congested blood vessels were perceived in connective tissue of pancreas. The islets of Langerhans showed cytoplasmic vacuolation (Figure 2A).

Group III (Nicotine - Lycopene treated group): pancreatic islets in this group have shown moderate amelioration in the number of islet cells and disappearance of congested blood vessels , hemorrhage and cytoplasmic vacuolation but still there is some degenerated cells and there is immature cells (Figure 3A).

Group IV (Nicotine – Folic acid treated group): pancreatic islets of nicotine& folic acid treated group (G IV) have appeared with well -defined outline nearly similar to control group, with centrally placed beta cells, and peripherally placed alpha cells. But there is few degenerated cells. (Figure 4A).

Group V (recovery group): pancreatic islets of recovery group showing some empty spaces between degenerated cells (E) and still there is small foci of hemorrhage (arrow), but there is disappearance of dilated congested bl.vessel (Figure 5A).

Masson trichrome stain

Little amount of collagen fiber was detected in pancreatic islets of Masson stained pancreatic sections of control group (Figure 1B)

Increased amount of collagen fibers around and inside the pancreatic islet and also around the blood vessel were noticed in the Masson stained pancreatic sections of (Nicotine treated group) (Figure 2B).

Minimal amount of collagen fibers inside and around the islets and around the blood vessel could be seen in Masson stained pancreatic sections of (Nicotine & Lycopene treated group) (Figure 3B).

Minimal amount of collagen fibers were seen in pancreatic islets of Masson stained pancreatic sections of (Nicotine + Folic acid treated group) (Figure 4B).

moderate amount of collagen fibers around the islets but increased amount around the blood vessels and in connective tissue septa were detected in Masson stained pancreatic sections of recovery group (Figure 5B).

Immuno-stained results

Anti-insulin

The immunohistochemical results revealed increased brown coloration that indicates positive cytoplasmic reaction, the strong positive reaction for anti-insulin in all the islet beta cells surrounded by thin rim of negatively immunostained alpha cells in the pancreas of the control group (G1) (Figure 1C).

Shrunken islet with few positive beta islet cells with large area of negatively immunostained cells were shown in pancreatic sections of the nicotine treated group (G2) (Figure. 2C).

Pancreatic islet of nicotine & lycopene treated group showing positive cytoplasmic expression for insulin in most of beta cells with moderate peripheral zone of negatively immunostained cells (Figure 3C) .

Pancreatic islet of nicotine & folic acid treated group revealed positive cytoplasmic expression for insulin in most of beta cells with moderate peripheral zone of negatively immunostained cells (Figure 4C) .

While pancreatic islet of recovery group showing shrunken islet with few positive beta islet cells with an area of negatively immunostained cells (Figure 5C).

INOs

The pancreatic islets of the control group revealed negative expression for iNOS (Figure 1D).

Pancreatic islets treated with nicotine revealed +ve immuno reaction for iNOS in the cytoplasm of peripheral and some central islet cells (Figure 2 D).

Examination of pancreatic sections of nicotine& lycopene treated group revealed very weak immuno reaction for iNOS in the cytoplasm of some peripheral islet cells (Figure 3D).

Immunohistochemical examination of iNOS stained pancreatic sections (Nicotine & Folic acid treated group) shown also a faint brown color in the cytoplasm of some of the beta cells nearly similar to control meaning very weak immuno reaction for iNOS in the cytoplasm of most of islet cells (Figure 4D).

INOs immunoexpression of the islets of pancreas of recovery group exposed dark brown color (positive immunoreaction) in the cytoplasm of central and peripheral islet cells (Figure 5D).

Morphometric results

The mean area % of collagen deposition for all groups has been represented in (Table 7, Histogram 7). There was significant increase in mean area% of collagen deposition ($P \leq 0.02$) in group II in comparing with groups I,III &IV. But area percentage of collagen deposition has increased in groups III & IV without significant difference in comparing with control group ($P > 0.05$). Also, area % of collagen deposition has decreased insignificantly in group IV in comparing with group III ($P > 0.05$). There was high significant increase in mean area percent of collagen deposition ($P \leq 0.02$) in group V in comparing with groups I,III & IV

The mean area percent of anti-insulin immuno-expression for all groups was represented in (Table 8, Histogram 8). The area percent of anti-insulin immunoreactivity has highly significantly decreased in group II in comparing with control group I ($P < 0.02$). In group IV the area percent of anti-insulin immunoreactivity has markedly improved with no significant difference in comparing with control group ($P > 0.05$) and it has highly significantly increased in comparing with group II ($P < 0.02$). Also, the area percent of anti-insulin immunoreactivity has highly significantly increased in group III in comparing with group II ($P < 0.02$) but it has decreased with significant difference when compared with control group ($P \leq 0.05$) and it has insignificantly decreased in comparing with group IV ($P > 0.05$). The area percent of anti-insulin immunoreactivity has highly significantly decreased in recovery group in comparing with groups I,III & IV ($p \leq 0.02$), but it has increased with no significant difference in comparing with group II.

The mean area percent of iNOS immuno-expression for all groups was represented in (Table 9, Histogram 9). There was significant increase in mean area% of iNOS ($P \leq 0.02$) in group II in comparing with groups I,III &IV. But area percentage of iNOS has increased in groups III & IV without significant difference in comparing with control group ($P > 0.05$) There was high significant increase in mean area percent of iNOS ($P \leq 0.02$) in group V in comparing with groups I,III & IV.

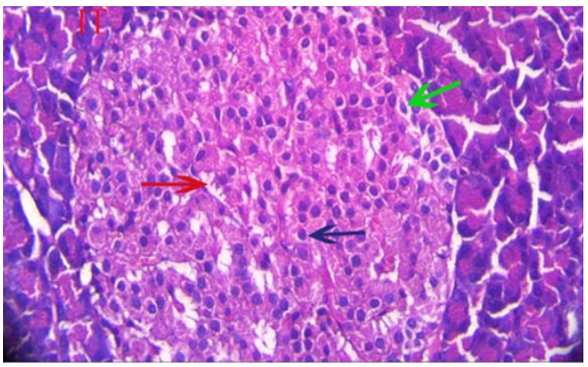


Fig. (1A): Photomicrograph of a section of the pancreas of control adult rat showing islets of pancreas (IT) with centrally placed beta cells (Black arrow) with rounded and pale nuclei and peripherally placed alpha cells (green arrow) with oval darkly stained nuclei, the islets appears as cords separated by blood capillaries (red arrow). (H&E X 400)

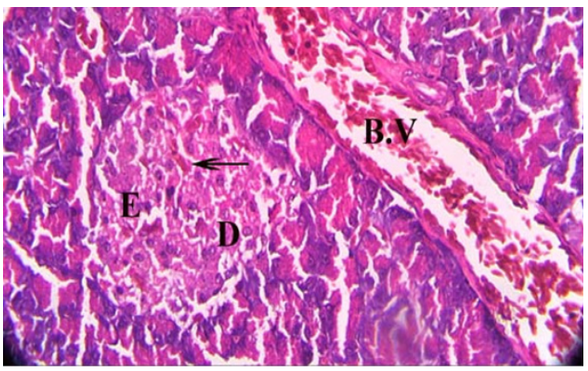


Fig. (2A): Photomicrograph of a section of adult rat pancreas treated with nicotine showing islets with ill-defined outline. There is decreased number of cells of islets. There is Empty spaces (E) between cords of cells and small foci of hemorrhage (arrow) in between some degenerated cells (D). Notice: dilated congested blood vessels (B.V). (H&E X 400)

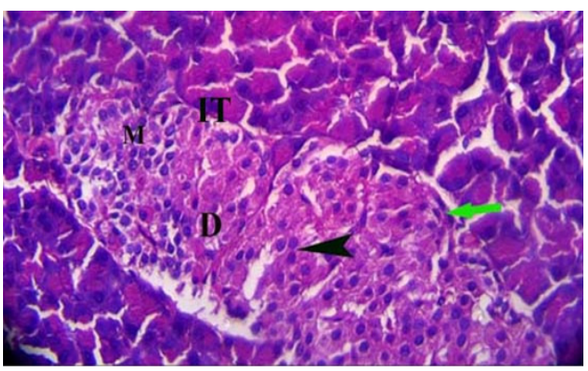


Fig. (3A): Photomicrograph of a section of adult rat pancreas of nicotine& lycopene treated group showing pancreatic islets with well -defined outline (IT) with centrally placed beta cells (arrow head), peripherally placed alpha cells (green arrow) , but there is some immature cells (M) and some degenerated cells (D). (H&E X 400)

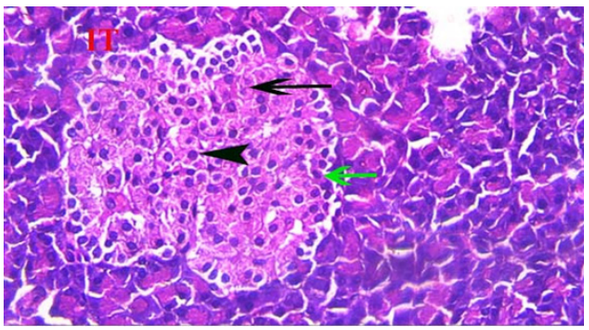


Fig. (4A): Photomicrograph of a section of adult rat pancreas of nicotine& folic acid treated group showing pancreatic islets with well -defined outline (IT) nearly similar to control group, with centrally placed beta cells (arrow head), and peripherally placed alpha cells (green arrow). Notice: few degenerated cells (black arrow). (H&E X 400)

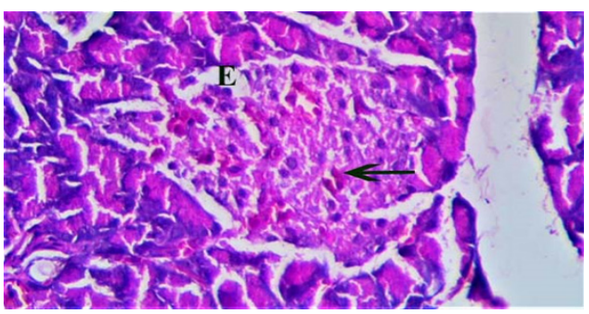


Fig. (5A): Photomicrograph of a section of adult rat pancreas of recovery group also showing some empty spaces between degenerated cells (E) and still there is small foci of hemorrhage (arrow) ,but there is disappearance of dilated congested bl.vessel. (H&E X 400)

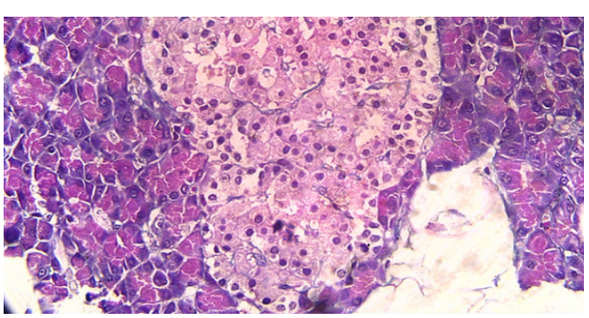


Fig. (1.B): Photomicrograph of a section of the pancreas of control adult rat showing minimal amount of collagen fibers inside the islets of pancreas. (Masson ,s trichrom X 400)

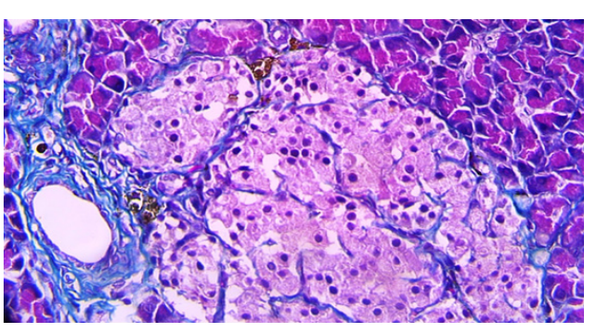


Fig. (2.B): Photomicrograph of a section of adult rat pancreas treated with nicotine showing increased amount of collagen fibers around and inside the pancreatic islet and also around the blood vessel. (Masson ,s trichrom X 400)

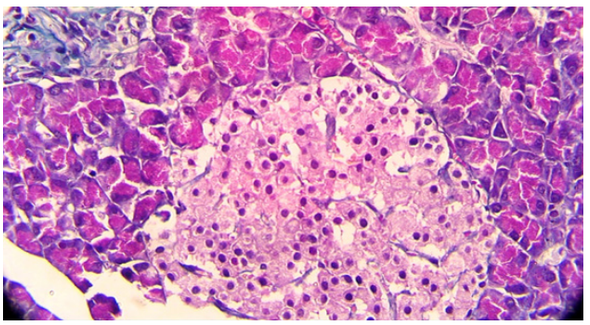


Fig. (3.B): Photomicrograph of a section of adult rat pancreas of nicotine& lycopene treated group showing minimal amount of collagen fibers inside and around the islets and around the blood vessel. (Masson ,s trichrom X 400)

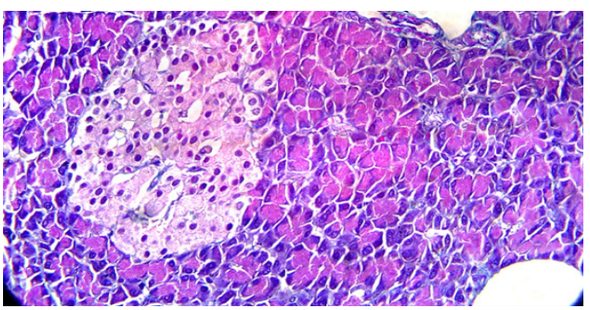


Fig. (4.B): Photomicrograph of a section of adult rat pancreas of nicotine& folic acid treated group showing minimal amount of collagen fibers inside the pancreatic islet. (Masson ,s trichrom X 400)

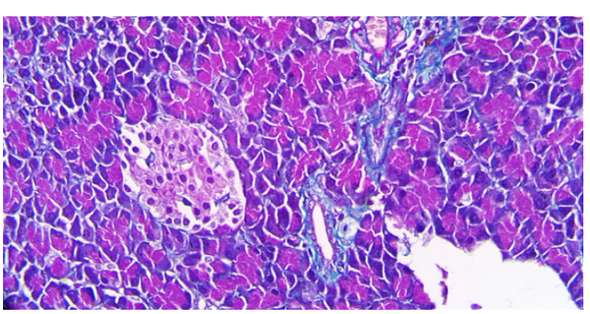


Fig. (5.B): Photomicrograph of a section of adult rat pancreas of recovery group also showing moderate amount of collagen fibers around the islets but increased amount around the blood vessels and in connective tissue septa. (Masson ,s trichrom X 400)

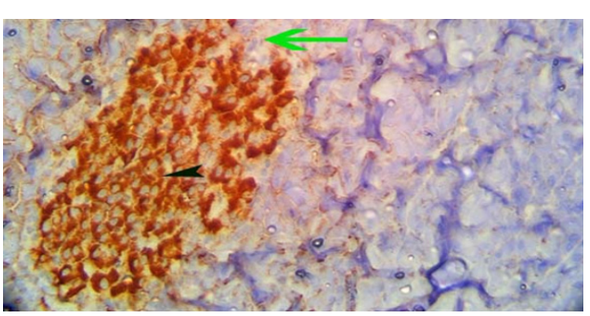


Fig. (1C): An immunostained photomicrograph of pancreatic islet of control group showing increased brown coloration that indicates positive cytoplasmic reaction, the positive reaction in all the islet beta cells (arrow head) surrounded by thin rim of negatively immunostained alpha cells (green arrow). (anti-insulin immune staining with counter stain hematoxylin X400)

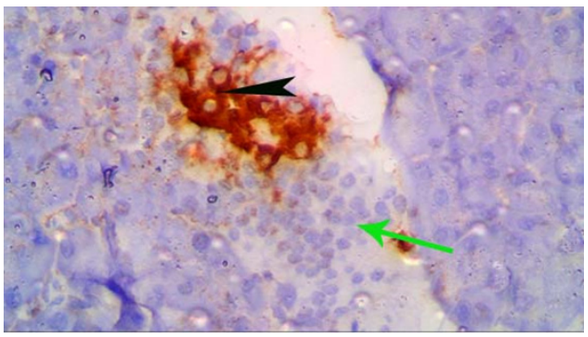


Fig. (2C): An immunostained photomicrograph of pancreatic islet of nicotine treated group showing shrunken islet with few positive beta islet cells (arrow head) with large area of negatively immunostained cells (green arrow). (anti-insulin immune staining with counter stain hematoxylin X400)

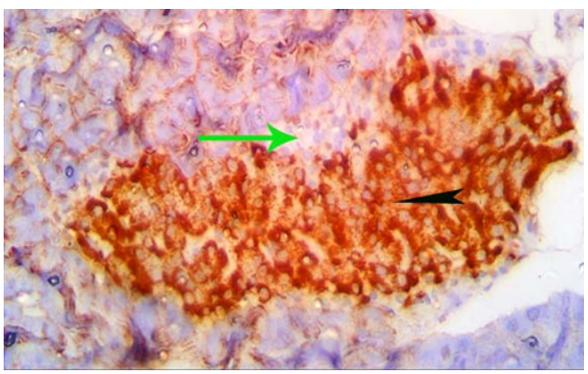


Fig. (3C): An immunostained photomicrograph of pancreatic islet of nicotine & lycopene treated group showing positive cytoplasmic expression for insulin in most of beta cells (arrow head) with moderate peripheral zone of negatively immunostained cells(green arrow). (anti-insulin immune staining with counter stain hematoxylin X400)

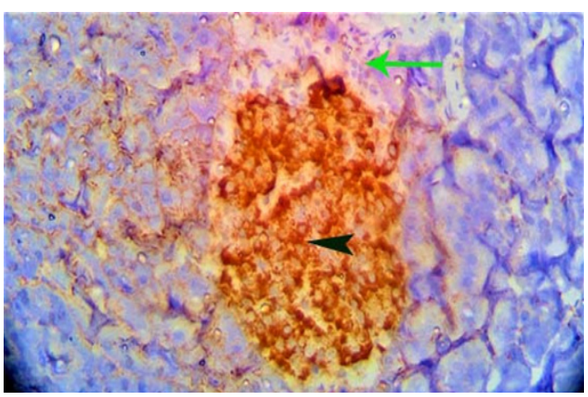


Fig. (4C): An immunostained photomicrograph of pancreatic islet of nicotine & folic acid treated group showing positive cytoplasmic expression for insulin in most of beta cells (arrow head) with moderate peripheral zone of negatively immunostained cells(green arrow). (anti-insulin immune staining with counter stain hematoxylin X400)

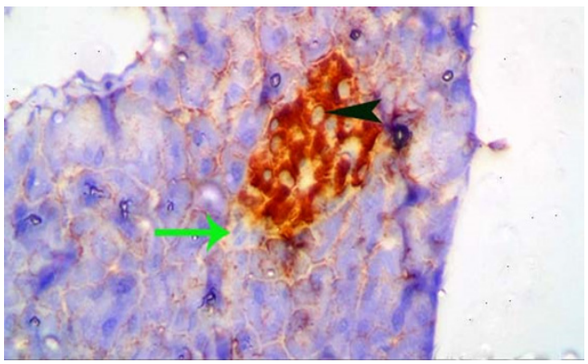


Fig. (5C): An immunostained photomicrograph of pancreatic islet of recovery group showing shrunken islet with few positive beta islet cells (arrow head) with an area of negatively immunostained cells (green arrow). (anti-insulin immune staining with counter stain hematoxylin X400)

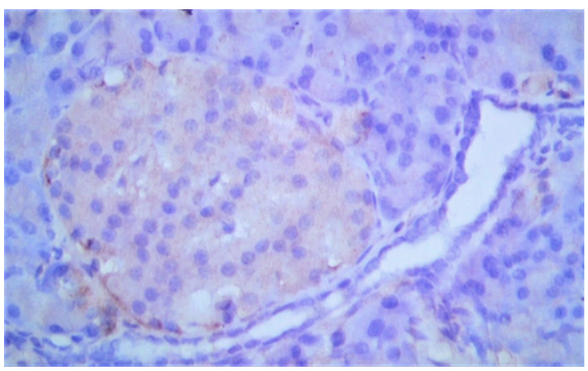


Fig. (1.D): Photomicrograph of a section of the pancreas of control adult rat showing islets of pancreas (IT) with a negative immuno reaction for iNOS in the cytoplasm of islets cells. (iNOS immunostaining with counter stain hematoxylin X 400)

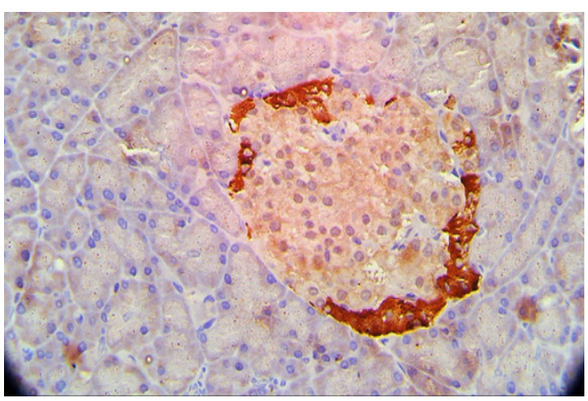


Fig. (2.D): Photomicrograph of a section of adult rat pancreas treated with nicotine showing positive immuno reaction for iNOS in the cytoplasm of peripheral and some central islet cells (iNOS immunostaining with counter stain hematoxylin X 400)

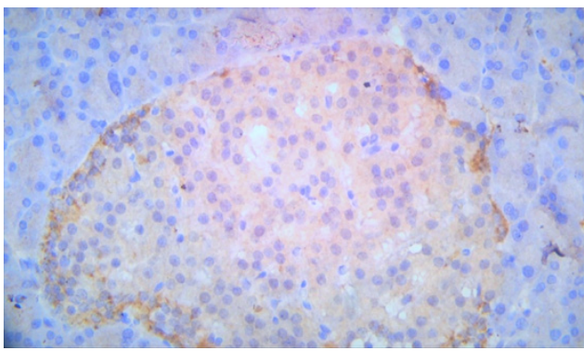


Fig. (3.D): Photomicrograph of a section of adult rat pancreas of nicotine& lycopene treated group showing very weak immuno reaction for iNOS in the cytoplasm of some prephiral islet cells (iNOS immunostaining with counter stain hematoxylin X 400).

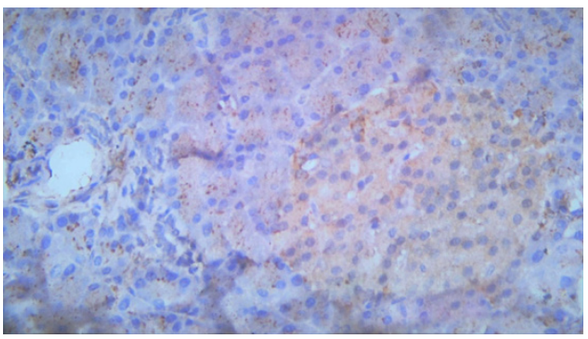


Fig. (4.D): Photomicrograph of a section of adult rat pancreas of nicotine& folic acid treated group showing very weak immuno reaction for iNOS in the cytoplasm of most of islet cells (iNOS immunostaining with counter stain hematoxylin X 400)

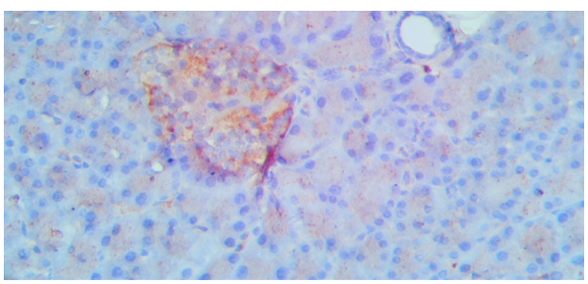


Fig. (5.D): Photomicrograph of a section of adult rat pancreas of recovery group showing positive immuno reaction for iNOS in the cytoplasm of central and peripheral islet cells (iNOS immunostaining with counter stain hematoxylin X 400)

Table 1: Showing mean values of GSH mmol/mg protein ± SD in the 5 groups

Mean mmol/mg protein ± SD	Group I	Group II	Group III	Group IV	Group V
GSH	58.47 ± 1.96	39.3 ± 8.03	53.7 ± 4.8	56.47 ± 1.15	39.27 ± 1.9
Significance ≤ 0.05	With groups II, III&V	With groups I,III & IV	With groups I, II & V	With groups II &V	With groups I,III & IV

Table 2: Showing mean values of MDA nmol/mg protein ± SD in the 5 groups

Mean nmol/mg protein ± SD	Group I	Group II	Group III	Group IV	Group V
MDA	68.3 ± 1.96	101.2± 8.03	76.9 ± 4.8	71.2 ± 1.15	95.3 ± 1.9
Significance ≤ 0.05	With groups II, &V	With groups I,III & IV	With groups II & V	With groups II &V	With groups I,III & IV

Table 3: Showing mean values of SOD U/mg protein ± SD in the 5 groups

Mean Unit /mg protein ± SD	Group I	Group II	Group III	Group IV	Group V
SOD	179.16 ± 3	143.4± 1.98	172.26 ± 2.2	176.6 ± 3.3	148± 1
Significance ≤ 0.05	With groups II, &V	With groups I,III & IV	With groups II & V	With groups II &V	With groups I,III & IV

Table 4: Showing mean values of FBG mg/dl ± SD in the 5 groups

Mean mg/dl ± SD	Group I	Group II	Group III	Group IV	Group V
FBG	75 ± 5	155 ± 5	89.3 ± 7.8	77.3 ± 4.5	140± 5
Significance ≤ 0.05	With groups II, &V	With groups I,III & IV	With groups II & V	With groups II &V	With groups I,III & IV

Table 5: Showing mean values of HbA1c ± SD in the 5 groups

Mean % ± SD	Group I	Group II	Group III	Group IV	Group V
HbA1c	4.8 ± 0.4	7.5 ± 0.4	5.7 ± 0.6	5.1 ± 0.25	7.2± 0.35
Significance ≤ 0.05	With groups II, &V	With groups I,III & IV	With groups II & V	With groups II &V	With groups I,III & IV

Table 6: Showing mean values of insulin µIU/ml ± SD in the 5 groups

Mean µIU/ml ± SD	Group I	Group II	Group III	Group IV	Group V
Insulin	12.3 ± 0.9	6.5 ± 1	9.9 ± 0.21	11. 6 ± 0.6	7.6 ± 0.8
Significance ≤ 0.05	With groups II, &V	With groups I,III & IV	With groups II & V	With groups II &V	With groups I,III & IV

Table 7: Showing mean values of area percent of collagen fibers deposition ± SD in the 5 groups

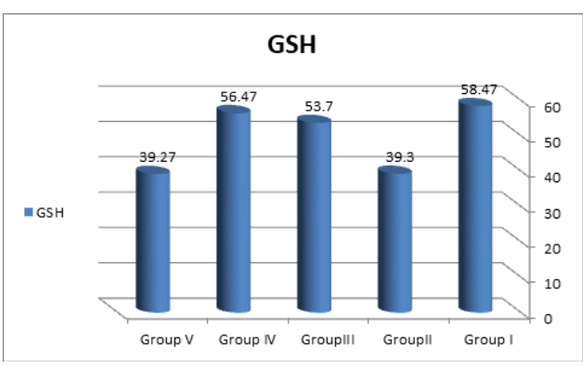
Mean % ± SD	Group I	Group II	Group III	Group IV	Group V
Masson%	4.53± 3.5	30.43± 4. 2	9.9± 1.1	5.1 ± 1.3	24.14±6.8
Significance ≤ 0.05	With groups II, &V	With groups I,III & IV	With groups II & V	With groups II &V	With groups I,III & IV

Table 8: Showing mean values of area percent immunoreactivity of anti-insulin ± SD in the 5 groups

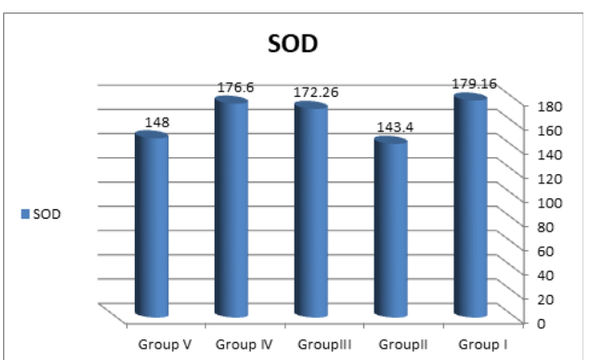
Mean % ± SD	Group I	Group II	Group III	Group IV	Group V
MA	25.54 ± 4.8	2.5± 1.7	18.46 ± 1.1	22.41 ± 2	4.7 ± 2.8
Significance ≤ 0.05	With groups II, &V	With groups I,III & IV	With groups II & V	With groups II &V	With groups I,III & IV

Table 9: Showing mean values of area percent immunoreactivity of iNOS ± SD in the 5 groups

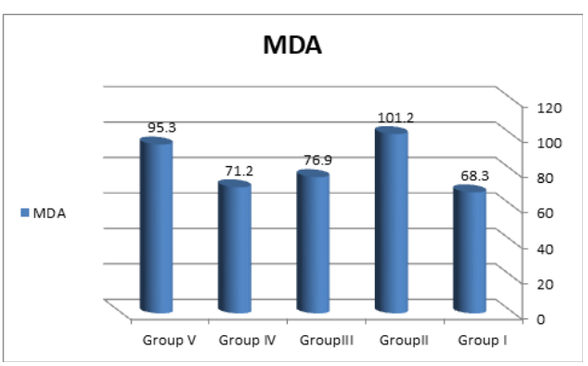
Mean % ± SD	Group I	Group II	Group III	Group IV	Group V
iNOS	5.11 ± 3.2	39.8 ± 15.2	8.9 ± 2.3	6.6 ± 1.4	32.5 ± 9.6
Significance ≤ 0.05	With groups II &V	With groups I,III & IV	With group II & V	With groups II &V	With groups I,III & IV



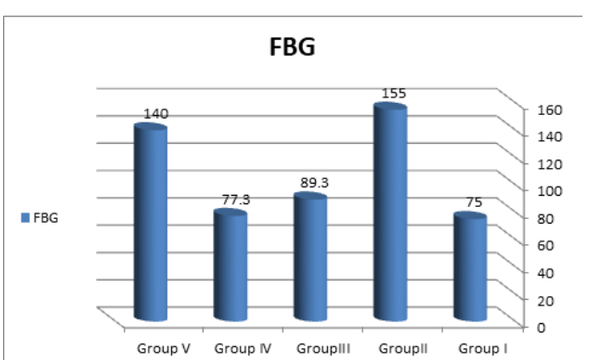
Histogram 1: Showing mean values of GSH mmol/mg protein in the 5 groups



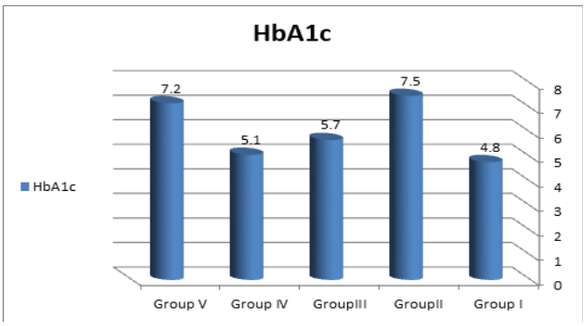
Histogram 3: Showing mean values of SOD U/mg protein in the 5 groups



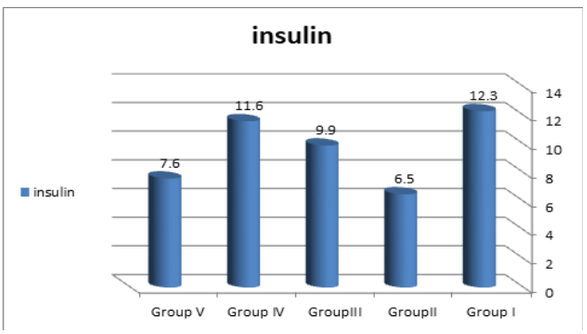
Histogram 2: Showing mean values of MDA nmol/mg protein in the 5 groups



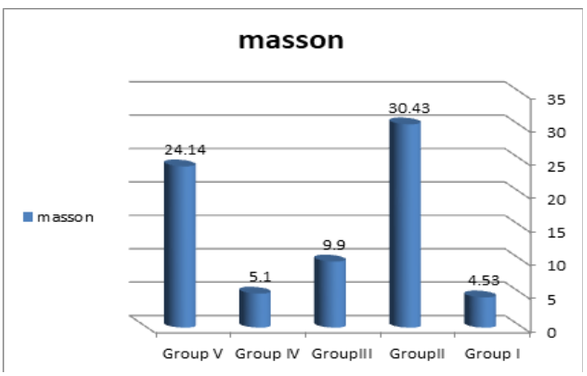
Histogram 4: Showing mean values of FBG mg/dl in the 5 groups



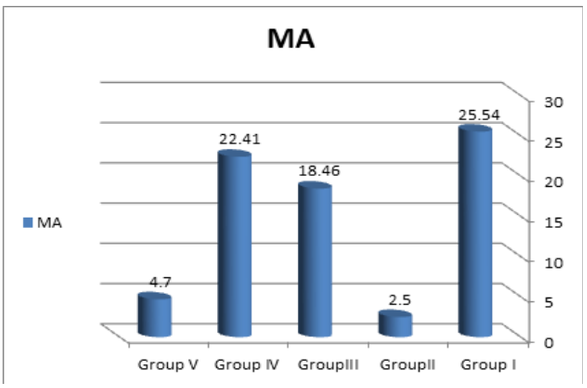
Histogram 5: Showing mean values of HbA1c in the 5 groups



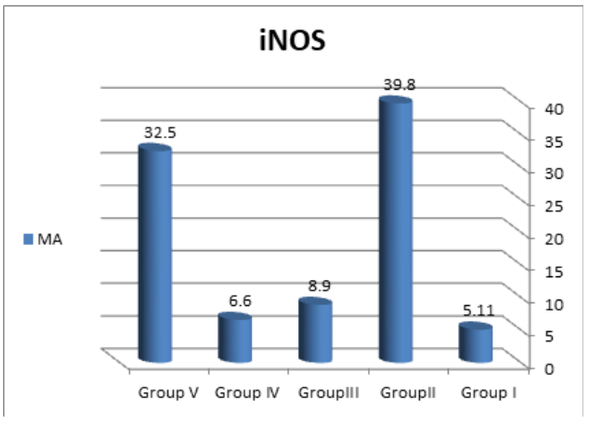
Histogram 6: Showing mean values of insulin µIU/ml in the 5 groups



Histogram 7: Showing mean values of area percent of collagen fibers deposition in the 5 groups



Histogram 8: Showing mean values of area percent immunoreactivity of anti-insulin in the 5 groups



Histogram 9: showing mean values of area percent immunoreactivity of iNOS in the 5 groups

DISCUSSION

Many people throughout the world consumed nicotine as tobacco and cigarette smoking. Though, side effects of nicotine were stated nearly on all organs and predominantly on endocrine glands however its impact on insulin production is still debated[25]. Additionally, nicotine exposure provokes ROS production in our body[5].

Several prospective mechanisms could clarify the useful impacts of lycopene intake on health, containing its potent antioxidant ability to resist to oxidative stress[26]

Lycopene, that gives tomatoes its red color, is a strong antioxidant and free oxygen radicals suppressor between other carotenoids. Antioxidant action of lycopene is due to anti-inflammatory efficacy in several experimental models of pancreatitis.[27]

Folic acid is one of the most important micronutrient which has an essential antioxidant activity.[28]

This work was intended to study the prophylactic impact of lycopene and folic acid against nicotine toxicity on pancreatic islets.

In our study the biochemical assay showed that the mean GSH, SOD & Insulin echelons were reduced significantly in nicotine exposed group compared to control group ($P \leq 0.05$), In nicotine & lycopene treated group serum levels of GSH & Insulin were significantly reduced in comparison with control group ($P \leq 0.05$) and insignificantly decreased as compared to nicotine & folic acid treated group ($P > 0.05$) but they were significantly increased compared with nicotine treated and recovery groups ($P \leq 0.05$).Serum levels of SOD in nicotine & lycopene treated group were insignificantly decreased as compared to control and nicotine & Folic acid treated groups ($P > 0.05$) but they were significantly increased when compared with nicotine treated and recovery groups ($P \leq 0.05$).

While mean MDA, FBG & HbA1c levels were increased significantly in nicotine treated group as

compared to control group ($P \leq 0.05$), while in nicotine & Folic acid treated group these serum levels were decreased significantly when compared to nicotine treated and recovery groups ($P \leq 0.05$) and insignificantly increased compared to control group ($P > 0.05$), in nicotine & lycopene treated group these levels were insignificantly increased In comparison to control and nicotine & Folic acid treated groups ($P > 0.05$) but they were significantly decreased when compared to nicotine treated and recovery groups ($P \leq 0.05$).

Previous studies showed that nicotine exposure significantly exhausted stores of GSH in pancreas, demonstrating that GSH was used as an antioxidant for the cleansing of poisonous oxygen products, thus nicotine exposure increases the liability of the pancreatic islets to oxidative injury. However, nicotine-induced decline in levels of GSH in pancreatic tissue were inverted by folic acid supplementation[5].

Also El-Sokkary *et al*, 2007 showed that poisonousness caused by nicotine is combined with GSH reduction, which is one of the critical composites for retaining integrity of the cell.[29]

Singh *et al*, 2020 showed that upon nicotine use, there was a reduction in SOD activities, which was increased markedly by of phytoene, phytofluene, and lycopene supplementation individually, enhanced degree of protection.[19]

Previous study explained that Folic acid decreased nicotine-induced oxidative damage with a simultaneous preservation of stores of GSH in the pancreatic tissue, including its antioxidant role in improving the tissue function. Chiefly, the findings on oxidant and antioxidant standards propose that cell toxicity caused by nicotine on pancreatic islets is due to the production of oxidative stress, which was improved by folic acid supplementation.[4]

Also Singh *et al*, 2020 elucidated that the rise in the SOD activities and restoration of the GSH stores might have added to the antioxidative power of lycopene . The mechanism by which the lycopene increase the levels of these antioxidant enzymes is not clear yet, but it is attractive to guess that these carotenoids in some way, by up regulating the expressions of the genes coding these antioxidant enzymes, rise the levels of SOD and GSH and consequently exhibit the antioxidative capacity of lycopene.[19]

Bhattacharjee *et al*, 2015 revealed that objective index of chronic glycemia, was found to be significantly increased in nicotine exposed group compared to the control group. In other nicotine exposed rats folic acid Supplementation significantly upturned nicotine-produced changes in HbA1C level.[4]

Also Mohamed *et al*, 2017 showed that there was a noteworthy increase in level of glucose in Nicotine exposed group.[30]

Clair *et al*, 2011 showed that smoking, and especially nicotine, results in a constant rise in blood glucose level and as well as in HbA1C level and lowers insulin level.[31]

Bhattacharjee *et al*, 2015 Indicated that in nicotine-treated group there was a significant decrease in the insulin levels which was increased insignificantly in the group supplemented with folic acid.[4]

Bhattacharjee *et al*, 2018 showed that activity of SOD was decreased in nicotine treated rats in comparison to control rats and that reduction in SOD activity was improved significantly by folic acid supplementation. Also in group treated with nicotine, there was a significant decline in level of GSH as compared to control group. While, GSH level was increased significantly in group treated with folic acid.[5]

Kalpana and Menon 2004 showed that secondary lipid peroxidation yields such as MDA may do similar poisonous impacts, which can extend and increase the damage initiated by primary free radical.[32]

Singh *et al*, 2020 clarified that there was a significant rise of MDA ($p < 0.01$) in nicotine-treated rats which were opposed by lycopene supplementation.[19]

Bhattacharjee *et al*, 2018 revealed that MDA output, an indicator of lipid peroxidation, was elevated prominently in nicotine treated rats. Folic acid Supplementation decreased nicotine-induced expulsion of MDA significantly.[5]

In the current study, H&E inspection of the pancreatic islets of group II (Nicotine treated group) showed islets with ill-defined outline. There is decreased number of cells of islets. There is empty spaces between cords of islets and small foci of hemorrhage in between some degenerated cells. Congested blood vessels were perceived in connective tissue of pancreas. The islets of Langerhans showed cytoplasmic vacuolation. Also showed increased amount of collagen fibers around and inside the pancreatic islet and also around the blood vessel were noticed in the Masson stained pancreatic sections. Shrunken islet with few positive beta islet cells with large area of negatively immunostained cells were shown in pancreatic sections stained with anti-insulin immunohistochemical stain. Pancreatic islets treated with nicotine revealed +ve immunoreaction for iNOS in the cytoplasm of peripheral & some central islet cells.

These findings are in agreement with Bruin *et al*, 2010 who stated that nicotine rises degeneration of islet beta cells in animal models treated with nicotine.[33]

Also Bhattacharjee *et al*, 2015 stated that hematoxylin and eosin stained slices of the nicotine treated pancreas showed islet cell injury, the islet was shrunken and the islet cell numbers were reduced.[4]

Also Mohamed *et al*, 2017 revealed that nicotine-treated rats showed cytoplasmic vacuolation inside the islets of Langerhans.[30]

A previous study Showed negative immune-reaction for insulin in β cells in injured islet. Inflammatory infiltration and advanced stage fibrosis of the pancreas were noticed around the islet[34].

Singh *et al*, 2020 explained the oxidative injury of the Pancreatic islet cells of nicotine-treated rats by the advanced level of Nitric Oxide generation and Reactive oxygen species production.[19]

Also Tong *et al*, 2020 showed that smoking impacts pancreatic B-cell function and survival as it reduced B-cell viability and proliferation[35].

Nicotine may stimulate oxidative stress in islet cells via the exhaustion of antioxidant and anti-oxidative enzymes and this is caused by Excessive Reactive oxygen species generation from mitochondria. This consequently leads to more apoptosis.[5]

In this work, examination of group III (nicotine + lycopene treated group) showed pancreatic islets with moderate amelioration in the number of islet cells and disappearance of congested blood vessels , hemorrhage and cytoplasmic vacuolation but still there is some degenerated cells and there is immature cells. Minimal amount of collagen fibers inside and around the islets and around the blood vessel could be seen in Masson stained pancreatic sections . Also pancreatic islet of nicotine & lycopene treated group showing positive cytoplasmic expression for insulin in most of beta cells with moderate peripheral zone of negatively immunostained cells. It revealed very weak immunoreaction for iNOS in the cytoplasm of some peripheral islet cells

Singh *et al*, 2020 Stated that hematoxylin & eosin stained pancreatic sections shown that nicotine exposure caused devastation of islet architecture with increased vacuolization of the pancreatic islets . This was opposed as the damage is detected to be decreased and rescue is started upon supplementation of lycopene.[19]

Supplementation of lycopene has opposed the nicotine induced pancreatic islet injury. It effectively opposed the rise in Reactive Oxygen Species production. It was also eligible for inhibiting the rise in pro-inflammatory cytokine levels and keeping the pancreatic islet architecture[19]. Also In another study, lycopene was found to work as an antidiabetic factor by decreasing formation of free radical[14]

Ozmen *et al*, 2016 stated that lycopene had a prophylactic effects on the insulin-secreting cells and the immunohistochemical examinations showed that lycopene had protective effects on islet beta cells that lycopene reduced pancreatic injury associated with diabetes Additionally, it raised serum insulin levels.[36]

In the current work, examination of pancreatic islets of nicotine& folic acid treated group (G IV) showed well -defined outline nearly similar to control group, with centrally placed beta cells, and peripherally placed alpha cells. But there is few degenerated cells. Minimal amount of collagen fibers were seen in pancreatic islets of Masson stained pancreatic sections .Also anti insulin stained sections revealed positive cytoplasmic expression for insulin in most of beta cells with moderate peripheral zone of negatively immunostained cells.

Folic acid supplementation gave defense against damaging impacts of nicotine in rat pancreatic islets.[4] Folic acid clears the cellular Reactive Oxygen Species and participates in saving of islet cells.[37]

Bhattacharjee *et al*, 2018 revealed that immune- staining of pancreatic sections exhibited that nicotine-treatment causes damage in rat islet cells which was improved by folic acid.[5]

In the current work, examination of pancreatic islets of Group V (recovery group) showed some empty spaces between degenerated cells and still there is small foci of haemorrhage, but there was disappearance of dilated congested blood vessels.

Mohamed *et al*, 2017 stated that After withdrawal of nicotine, destructive effect on some islet cells persisted in the form of cytoplasmic vacuolation, increased numbers of empty β cell granules and pyknotic cells.[30]

CONCLUSION

The use of lycopene during the period of nicotine exposure is considered to have a protecting influence on pancreatic islets mean while the use of folic acid has a more protection than lycopene

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

التأثير الوقائى لمكملات الليكوبين وحمض الفوليك ضد سمية النيكوتين على جزر البنكرياس فى ذكور الجرذان البيضاء البالغة:دراسة بيوكيميائية ونسيجية مناعية

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مقدمة: تشير التقارير إلى وجود اختلال وظيفي في الجزر البنكرياسية وارتفاع مستويات السكر في الدم أثناء الصيام في الفئران عند تعرضها للنيكوتين و يُعتقد أن الليكوبين له دور محتمل كمضاد أكسدة فعال في الوقاية من الأمراض المزمنة المرتبطة بالإجهاد التأكسدي. حمض الفوليك هو فيتامين ب قابل للذوبان في الماء وضروري للتمثيل الغذائي للأحماض الأمينية

الهدف من الدراسة: تهدف هذه الدراسة إلى استكشاف آثار تسمم النيكوتين على جزر البنكرياس والتأثير الوقائي لمكملات الليكوبين وحمض الفوليك.

المواد والطرق: تم فصل خمسين من ذكور الجرذان البالغة الأصحاء إلى خمس مجموعات متساوية. المجموعة الضابطة , المجموعة المعالجة بالنيكوتين حيث تم حقن الجرذان داخل الصفاق بالنيكوتين ٣ ملجم / كجم يوميا لمدة ٣ اسابيع, المجموعة المعالجة بالنيكوتين + الليكوبين حيث تلقت الجرذان الليكوبين بجرعة ١٠ مجم / كجم من وزن الجسم. يومياً مع علاج النيكوتين لمدة ٣ أسابيع. المجموعة المعالجة بحمض الفوليك والنيكوتين حيث تم حقن الجرذان بالنيكوتين مثل المجموعة الثانية وحمض الفوليك عن طريق الفم بجرعة ٣٦ ميكروجرام / كجم. من وزن الجسم ومجموعة االتعافي التي بقيت الفئران بها لمدة شهر واحد بعد ٣ أسابيع من حقن النيكوتين. بعد ذلك تم فحص أنسجة البنكرياس من أجل التغيرات النسيجية المرضية والهيستوكيميائية المناعية.

النتائج: أظهرت المجموعة التي عولجت بالنيكوتين أن جزر البنكرياس متحلله والشكل الخارجي غير محدد. كما توجد العديد من ألياف الكولاجين داخل وحول جزر البنكرياس في شرايح البنكرياس المصبوغة بصبغة الماسون .وجود نشاط مناعي قوي لـ INO في حين انخفاض التعبير المناعي المضاد للأنسولين. يقلل الليكوبين وحمض الفوليك من التأثير السام للنيكوتين على جزر البنكرياس ، لكن حمض الفوليك أظهر انخفاضًا كبيرًا في ألياف الكولاجين والنشاط المناعي ل INOs و زيادة ملحوظة في التعبير المناعي المضاد للأنسولين مقارنة بتلك الموجودة في مجموعة الليكوبين.

الاستنتاج: يعتبر استخدام الليكوبين خلال فترة حقن النيكوتين له تأثير وقائي على جزر البنكرياس وفي الوقت نفسه ، فإن استخدام حمض الفوليك له حماية أكثر من الليكوبين