Original Article

Effect of Energy Drink on the Pancreas of Adult Male Albino Rat and the Possible Protective Role of Avocado Oil. Histological and Immunohistochemical Study

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ABSTRACT

Introduction: Energy drinks are widely used among youth as boosters to increase their concentration. Many Health disorders are associated with energy drinks. Avocado oil is a natural oil extracted from avocado fruit and possess an antioxidant and anti-inflammatory effects.

Aim of the Work: This work was conducted to investigate the effect of energy drink on the histological structure of the pancreas of adult male albino rat and to evaluate the possible protective role of avocado oil.

Materials and Methods: Forty adult male albino rats were divided equally into 4 groups; control group, avocado oil-treated group (0.4 mg/100 gm body weight), energy drink-treated group (1.5 mg/100 gm body weight) and both avocado oil and energy drink treated group. All rats were orally administrated once daily for 4 weeks. Pancreatic specimens were processed for light and electron microscopy. Immunohistochemical study was performed using insulin and caspase-3 antibodies.

Results: Energy drink-treated group revealed focal disturbed architecture of the exocrine and endocrine parts of pancreas. The exocrine pancreas appeared with cytoplasmic vacuolations and deeply stained nuclei of the acinar cells in addition to congested blood vessels. Ultrastructural examination revealed both acinar and islet cells with cytoplasmic vacuolations, swollen mitochondria with disrupted cristae, dilated rough endoplasmic reticulum in addition to shrunken condensed nuclei with dilated perinuclear cisternae. The immunohistochemical study showed a significant decrease in the insulin immunoreaction of β cells associated with a significant increase in caspase-3 immunoreaction in acinar and islet cells. In contrast, minimal changes were observed in rats treated concomitantly with avocado oil and energy drink with a non-significant change in the immunohistochemical reaction.

Conclusion: Energy drink induced structural changes in the pancreas of rats and the concomitant administration of avocado oil could to some degree ameliorate such changes.

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Key Words: Active caspase-3; energy drink; insulin; avocado oil; pancreas.

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INTRODUCTION

The energy drink is a special type of drink that gives energy by the addition of many energy enhancing ingredients or boosters, most notably caffeine and other ingredients including simple sugars (such as glucose and fructose), amino acids (such as taurine and carnitine) and artificial sweeteners (such as glucuronolactone) in addition to herbal supplements and vitamin B complex and other plant -based stimulants[1].

Nowadays, the energy drinks are widely used among youth and athletes all over the world to increase their physical performance, alertness, attention and concentration[2,3,4]. As the popularity of energy drinks continues to increase, their potential adverse effects become a major point of concern. Many studies revealed their side effects on liver and kidney in addition to their adverse effects on hematopoietic, cardiovascular and nervous systems[5].

Avocado is a tropical and subtropical fruit that is distributed worldwide. Its pulp is rich in lipids which makes it a source for oil extraction. There is a growing interest in avocado oil due to its high content of monounsaturated and polyunsaturated fatty acids in addition to many bioactive substances such as carotenoids, polyphenols, phytosterol, vitamins as vitamin E, B and tocopherols[6,7]. Moreover, Avocado oil has been widely used due to its antioxidant, anti-inflammatory, anti-bacterial, anti-fungal, and immune-modulatory effects. Moreover, it has a beneficial role in lowering the blood glucose level[8,9].

Based on the previous data, this study was designed to investigate the effect of the energy drink on the pancreas of adult male albino rat and to evaluate the possible protective role of avocado oil supplementation using different histological and immunohistochemical methods.

MATERIALS AND METHODS

This study was performed on 40 adult male albino rats weighing 180–200 grams each. All the animals were kept in suitable clean properly ventilated cages under similar conditions and were fed on a similar laboratory diet and

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water. They were adapted to their environment one week before starting the experiment. The experiment was approved by the Local Ethics Committee of Faculty of Medicine, Tanta University, Egypt.

The animals were randomly divided into 4 equal groups (10 rats each):

Group A (Control group): Animals of this group received no treatment for the same periods as the experimental animals. They were used to study the normal histological structure of the pancreas.

Group B (Avocado oil group): Animals of this group were orally administered 0.4 ml/100 gm body weight avocado oil using a gastric tube once daily for 4 weeks[10]. Avocado oil was purchased from EL Hawag company for extraction and packing of natural oils, Cairo, Egypt.

Group C (Energy drink-treated group): Animals of this group were orally administered 1.5 ml/100 gm body weight of energy drink using a gastric tube once daily for 4 weeks[5]. Energy drink (red bull) was obtained from a commercial source.

Group D (Avocado oil & energy drink-treated group): Animals of this group were co-administered both avocado oil and energy drink at the same dose, route and duration as in groups B&C respectively.

24 hours after the last dose, the animals were euthanized by an intra-peritoneal injection of sodium thiopental (40 mg/kg)[11]. The abdominal wall of each rat was opened through a mid-ventral incision along the entire length of the abdominal cavity and the pancreas was extracted. The rat pancreas is divided into duodenal, gastric and splenic segments. Splenic segment contains higher number of islets than in other regions[12]. Specimens mainly from the splenic segment of pancreas were obtained and processed for light and transmission electron microscopy.

For light microscopy

Pancreatic specimens were immersed in 10% neutral-buffered formalin, washed, dehydrated, cleared and embedded in paraffin. Pancreatic sections of 5µm thickness were stained with haematoxylin & eosin (H&E)[13].

For immunohistochemical staining

Pancreatic sections of 5 µm thickness were deparaffinized, rehydrated and washed with phosphate buffered saline (PBS) then incubated with 10% normal goat serum in PBS. Then, the sections were incubated overnight in a humid chamber at 4°C with the primary antibodies; rabbit polyclonal antibody anti-insulin (ab63820, Abcam, Cambridge, Massachusetts, USA) and rabbit polyclonal antibody anti-active caspase-3 (ab2302; Abcam, Cambridge, Massachusetts, USA), then they were incubated with biotinylated goat anti-rabbit IgG for 60 min at room temperature then with a streptavidin–biotin–horseradish peroxidase complex for another 60 min. The immunoreaction was visualized using 3,3′-diaminobenzidine (DAB) hydrogen peroxide chromogen and the sections were counterstained with Mayer's haematoxylin. The negative control sections were performed by excluding the primary antibodies[14]. Positive controls for insulin were the human & rat pancreas. Positive controls for active caspase-3 were camptothecin treated Jurkat cells. The insulin immune-stained pancreatic sections were considered positive when expressing clear evident brown cytoplasmic coloration. The active caspase-3- immune-stained pancreatic sections were considered positive when expressing clear evident brown nuclear and/or cytoplasmic coloration.

For transmission electron microscopy

Pancreatic specimens were cut into small pieces and fixed in 4% phosphate buffered gluteraldehyde (0.1 M, pH 7.3), post-fixed with 1% phosphate-buffered osmium tetroxide, and then dehydrated in ascending grades of ethanol. After being immersed in propylene oxide, the specimens were then embedded in epoxy resin mixture. Semithin sections (1µm thick) were stained with 1% toluidine blue and examined by light microscope to select suitable areas for ultrathin sectioning[15]. Ultrathin sections (80-90nm) were stained with uranyl acetate and lead citrate, to be examined by JEOL-JEM-100 transmission electron microscope (Tokyo, Japan) at the Electron Microscopic Unit, Faculty of Medicine, Tanta University, Egypt.

Morphometric analysis

A Leica light microscope (DM500, Swizterland) coupled to a Leica digital camera (ICC50, Swizterland) was used for image acquisition and the software "ImageJ" (version 1.48v National Institute of Health, Bethesda, Maryland, USA) was used for image analysis. Ten different non-overlapping randomly selected fields from each section were examined to quantitatively evaluate:

1. The mean color intensity of insulin immunohistochemical reaction (in DAB-stained sections at a magnification power of 400).
2. The mean area percentage of positive insulin immunohistochemical reaction (in DAB-stained sections at a magnification power of 400).
3. The mean color intensity of active caspase-3 immunohistochemical reaction (in DAB-stained sections at a magnification power of 1000).
4. The mean number of zymogen granules (in ultrathin sections at a magnification power of 2000)

Statistical analysis

All the previous data were statistically analyzed using SPSS software version 13 (SPSS Inc., Chicago, IL, USA), then compared by one way analysis of variance (ANOVA) test followed by Tukey's test to compare different groups with control group. The results were expressed as mean ± standard deviation (SD). The differences were considered statistically significant if probability value $P < 0.05$ and highly significant if $P < 0.001$[16].

RESULTS

In the present work, no deaths were reported during the experimental period.

Light microscopic results

H&E staining

Examination of pancreatic sections from both control group (A) and avocado oil treated group (B) showed similar histological results. The exocrine part of pancreas was formed of numerous pancreatic acini that were closely packed and separated from each other by very little connective tissue septae containing blood capillaries. Each pancreatic acinus had a very narrow acinar lumen, and it was formed of pyramidal shaped acinar cells. The cytoplasm of the acinar cells appeared with an intense basal basophilia and apical acidophilia. The nuclei of the acinar cells were basal, rounded and had prominent nucleoli. An intralobular duct lined with simple cubical epithelium appeared in between the pancreatic acini (Figures 1,2A,2B). The endocrine part of pancreas (islets of Langerhans) appeared lightly stained areas among the deeply stained pancreatic acini. The islets cells were rounded or polygonal in shape and arranged in anastomosing cords, and blood capillaries were present in between them. The cytoplasm of the islet cells was lightly acidophilic, and their nuclei were pale stained having prominent nucleoli (Figure 3).

The examined pancreatic sections from energy drink-treated group (C) revealed focal disrupted pancreatic architecture. The affected pancreatic acini lost their basal basophilia and apical acidophilia and appeared widely separated by thick connective tissue septae (Figure 4). Multiple cytoplasmic vacuoles appeared in the affected acinar cells. Moreover, the nuclei of the affected acinar cells were peripheral, flattened and darkly stained. In addition, homogenous acidophilic material was noticed between the pancreatic acini (Figure 5). Some blood capillaries appeared dilated with extravasation of red blood cells in between the acini (Figure 6). Furthermore, dilated congested blood vessels appeared with detached endothelium in addition to marked perivascular mononuclear cellular infiltration (Figure 7). Regarding the pancreatic ducts, they were dilated with retained secretion, and were lined with flattened epithelial cells (Figure 8). As regards the endocrine part of pancreas, the pancreatic islets, some of them appeared having irregular outlines with disturbed architecture. Multiple cytoplasmic vacuoles appeared in the affected islets cells giving them a foamy appearance. The nuclei of the affected islets cells appeared small and darkly stained. Moreover, dilated congested blood capillaries were noticed in between the islet cells (Figures 9,10,11).

The examined pancreatic sections of avocado oil & energy drink treated group (D) showed regression of some of the microscopic lesions. As regards the exocrine part of pancreas, most of pancreatic acini resembled the normal structure, but few of them appeared having darkly stained nuclei with perinuclear halos (Figure 12). Regarding the endocrine part, most of the islet cells appeared more or less normal having rounded nuclei and pale cytoplasm. However, few islets cells appeared having vacuolated cytoplasm and darkly stained nuclei (Figures 13A,13B).

Immunohistochemical staining against insulin

Insulin-immunohistochemical stained sections from control group (A) & avocado oil treated group (B) revealed a strong positive cytoplasmic reaction that was in the form of a brownish coloration of β cells (Figure 14A). While sections obtained from energy drink-treated group (C) showed a weak positive cytoplasmic reaction of β cells (Figure 14B). However, sections from avocado oil & energy drink treated group (D) illustrated a strong positive cytoplasmic reaction of β cells (Figure 14C).

Morphometric analysis of the mean color intensity and mean area percentage of insulin immunoreaction of β cells showed statistically highly significant decrease in the energy drink treated group (C) as compared with control group (A) and avocado oil treated group (B), whereas avocado oil & energy drink-treated group (D) revealed a non-significant change compared with control group. All these data were demonstrated in (Table 1, Histograms 1,2).

Immunohistochemical staining against active caspase III

Active caspase III-immunohistochemical stained sections from control group (A) & avocado oil treated group (B) revealed negative reaction in the acinar cells as well as the islet cells (Figures 15A,15B). While sections obtained from energy drink-treated group (C) showed a strong positive cytoplasmic reaction in many acinar cells (apical cytoplasmic) and islet cells (Figures 16A,16B). However, sections from avocado oil & energy drink treated group (D) illustrated a weak positive cytoplasmic reaction in few acinar cells and islet cells (Figures 17A,17B).

Morphometric analysis of the mean color intensity of active caspase III immunohistochemical positive reaction revealed a highly significant increase in energy drink-treated group (C) compared with control group (A) and avocado oil treated group (B), whereas avocado oil & energy drink-treated group (D) revealed non-significant change as compared with control group. All these data were illustrated in (Table 1, Histogram 3).

Electron microscopic results

The examined ultrathin sections of pancreatic tissue from both control group (A) and avocado oil group (B) showed similar findings. The acinar cell appeared having polarized cell features. The apical part contained numerous electron dense zymogen granules and Golgi apparatus. Moreover, the apical cell membrane had numerous microvilli projecting into a narrow lumen. The basal part of the acinar cell was occupied by parallel cisternae of rough endoplasmic reticulum (RER) surrounding the nucleus, and the mitochondria were located in between the

RER cisternae. The nucleus of the acinar cell was basal, rounded and euchromatic with prominent nucleolus. The intercellular spaces appeared as narrow electron lucent spaces separating the adjacent cells. Moreover, junctional complexes appeared joining the acinar cells together at their lateral walls in the apical domain of the cells (Figures 18,19).

Concerning the islets of Langerhans, they appeared with normal ultrastructure of their cells. The beta (β) cells had rounded or slightly oval nuclei with regular contour, and their cytoplasm contained secretory granules with an electron dense core surrounded by a wide electron lucent halo. Multiple mitochondria, RER and Golgi apparatus were seen scattered in their cytoplasm. The alpha cells appeared having homogenous electron dense granules (Figures 20,21).

The Examined ultrathin sections of pancreatic tissue from energy drink- treated group (C) showed many acinar cells with widening of intercellular spaces as well as the acinar lumen. Some acinar cells appeared having short microvilli and others showed disappearance of microvilli (Figure 22). Some acinar cells contained many cytoplasmic vacuoles, and revealed swollen mitochondria with destroyed cristae, dilated RER and perinuclear cisternae. Moreover, some of zymogen granules appeared with heterogenous contents and others showed peripheral dissolution of their contents. Few acinar cells showed depletion of zymogen granules and others showed basal migration of some zymogen granules. Regarding the nuclei of acinar cells, they appeared irregular, shrunken with condensed chromatin and surrounded with perinuclear dilated vacuolar spaces (Figures 23,24,25).

Regarding the islets of Langerhans of this group, the β cells appeared having cytoplasmic granules with small electron dense cores and surrounded by wide electron lucent halos. In addition, multiple cytoplasmic vacuoles were present. Moreover, some nuclei of β cells appeared with irregular outlines, and others were shrunken with dilated perinuclear cisternae. Moreover, some β cells showed swollen mitochondria, dilated Golgi apparatus and autophagic vacuoles (Figures 26,27,28).

As regards the avocado oil & energy drink-treated group (D), the examined ultrathin sections illustrated partial restoration of the normal architecture of exocrine and endocrine parts of the pancreas. Most of acinar cells contained numerous apical cytoplasmic zymogen granules, but few of them contained few zymogen granules. Most of cells showed apparently normal RER and mitochondria, but few of them showed a mildly dilatated RER (Figure 29). The β cells of the endocrine pancreas appeared more or less normal and having many cytoplasmic granules, but few granules appeared with a wide halo space around their cores. Moreover, few β cells contained few cytoplasmic vacuoles and dilated Golgi apparatus (Figure 30).

Morphometric analysis of the mean number of zymogen granules revealed a statistically highly significant decrease in the mean number of zymogen granules in energy drink-treated group (C) compared with control group (A) and avocado oil treated group (B), whereas avocado oil & energy drink-treated group (D) revealed non-significant change as compared with control group. All these data were illustrated in table (1) and histogram (4).

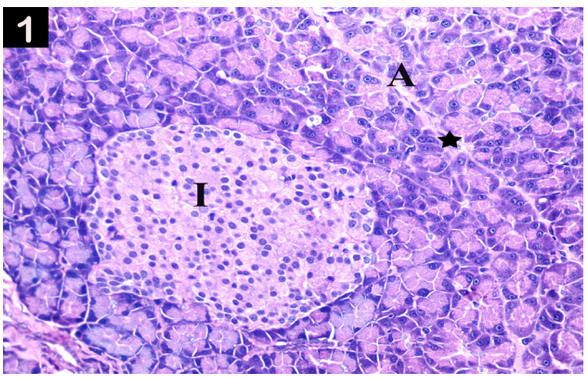


Fig. 1: A photomicrograph of a pancreas from control group showing a part of pancreatic lobules with closely packed exocrine acini (A). A pale stained islet of Langerhans (I) is seen in-between the acini. Notice the thin interlobular connective tissue septae (asterisks) (H&E X 400)

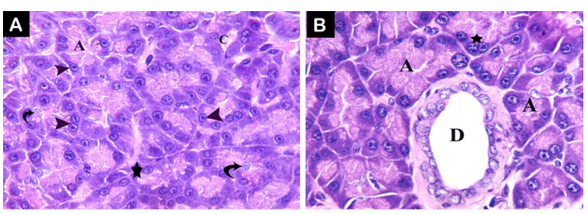


Fig. 2: A photomicrograph of pancreas from control group. (A): showing closely packed pancreatic acini (A), the acinar cells are pyramidal in shape having intense basal basophilia and apical acidophilia, their nuclei are basal, rounded and having prominent nucleoli (arrow heads). Normal blood capillary (C) and thin interlobular connective tissue septae are seen. Note: Nuclei of centroacinar cells are seen inside the lumen of some acini (curved arrow). (B): showing an intralobular duct (D) lined with cubical epithelium (A). Note: binucleated acinar cells (asterisk) are noticed. (H&E X 1000)

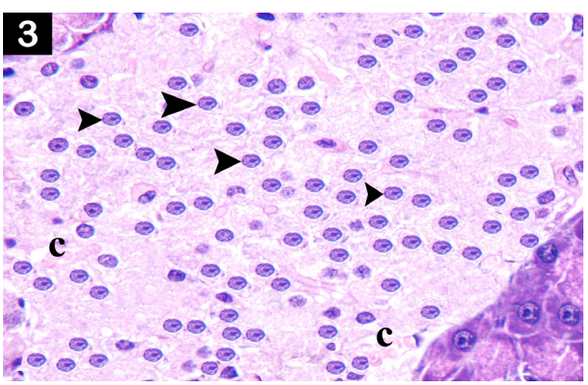


Fig. 3: A photomicrograph of a pancreas from control group showing an islet of Langerhans composed of cords of cells separated by blood capillaries (C). The cells are rounded in shape. Their cytoplasm appears pale acidophilic and the nuclei are pale stained with prominent nucleoli (arrow heads). (H&E X 1000)

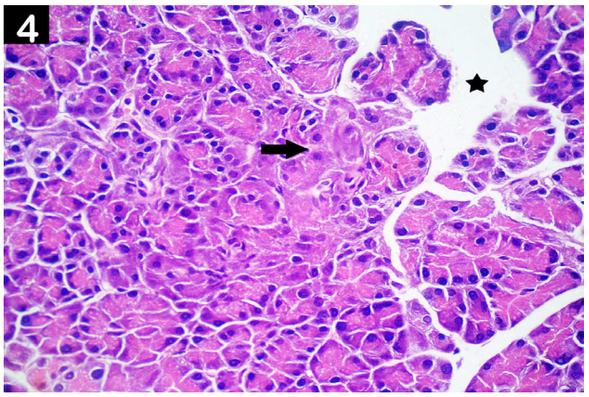


Fig. 4: A photomicrograph of a pancreas from energy drink treated group showing focal loss of normal architecture of pancreatic acini that appear widely separated by thick connective tissue septae (asterisk). The acini lost their basal basophilia and appear having acidophilic cytoplasm (thick arrow). (H&E X 400)

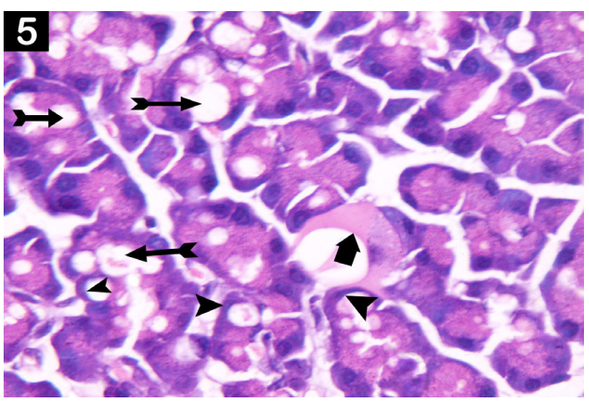


Fig. 5: A photomicrograph of a pancreas from energy drink-treated group showing many acinar cells with cytoplasmic vacuoles (bifid arrows) and peripheral flattened darkly stained nuclei (arrow heads). Notice the homogenous acidophilic material in-between the pancreatic acini (thick short arrow). (H&E X 1000)

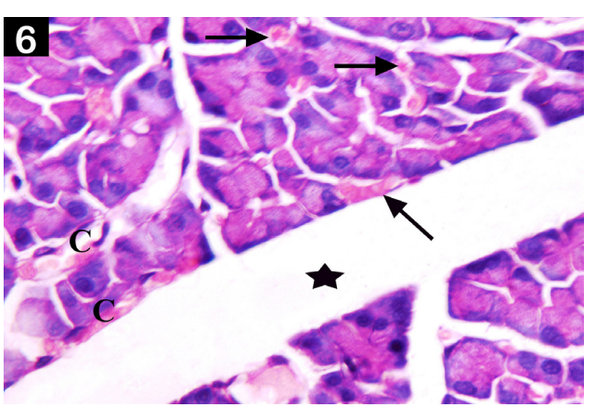


Fig. 6: A photomicrograph of a pancreas from energy drink-treated group showing dilated blood capillaries (C) with extravasation of red blood cells between the pancreatic acini (thin arrows). Notice the wide interlobular space (asterisk). (H&E X 1000)

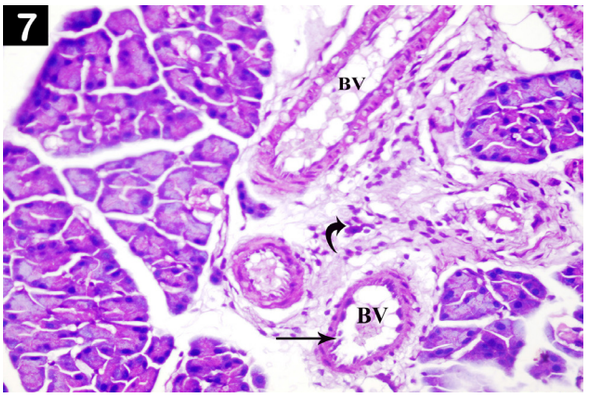


Fig. 7: A photomicrograph of a pancreas from energy drink-treated group showing dilated blood vessels (BV) with detached endothelium (thin long arrow) and marked mononuclear cellular infiltration (curved arrow). (H&E X 400)

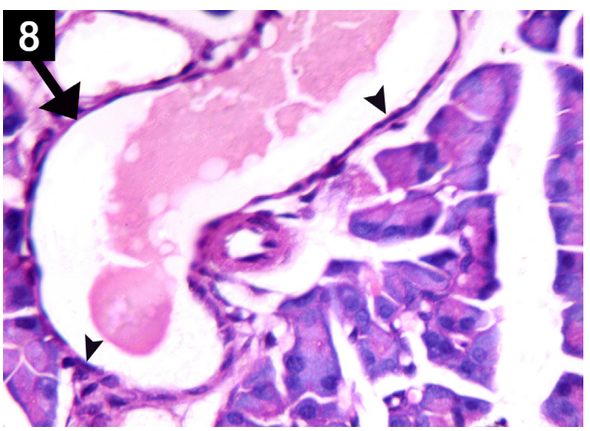


Fig. 8: A photomicrograph of a pancreas from energy drink-treated group showing dilated pancreatic duct (arrow) with retained acidophilic secretion. Notice the flattened lining epithelial cells (arrow heads) of the dilated duct. (H&E X 1000)

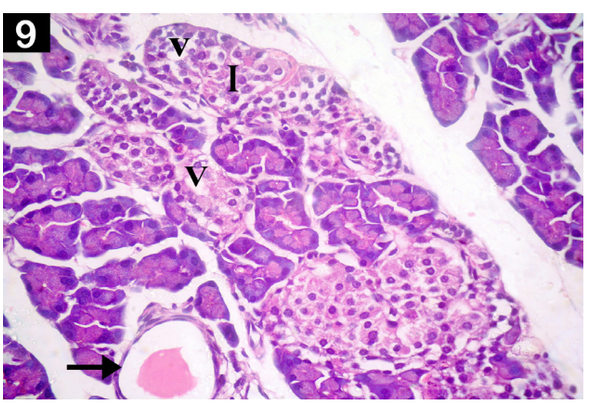


Fig. 9: A photomicrograph of a pancreas from energy drink-treated group showing an islet of Langerhans (I) with irregular outlines and disturbed architecture. Some islet cells contain many cytoplasmic vacuoles (V). Notice dilated pancreatic duct (arrow) with retained acidophilic secretion. (H&E X 400)

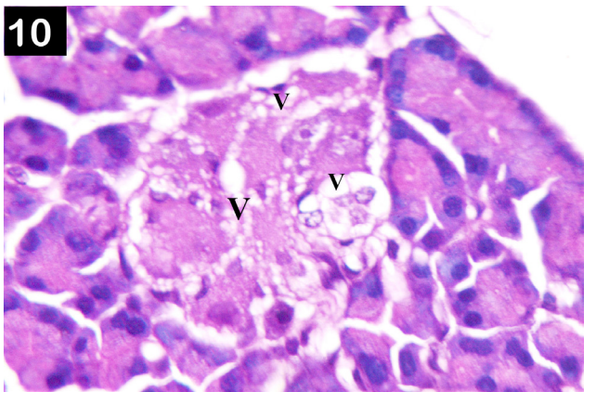


Fig. 10: A photomicrograph of a pancreas of energy drink-treated group showing loss of normal architecture of islet cells that appear having multiple cytoplasmic vacuoles (V) giving them a foamy appearance. (H&E X 1000)

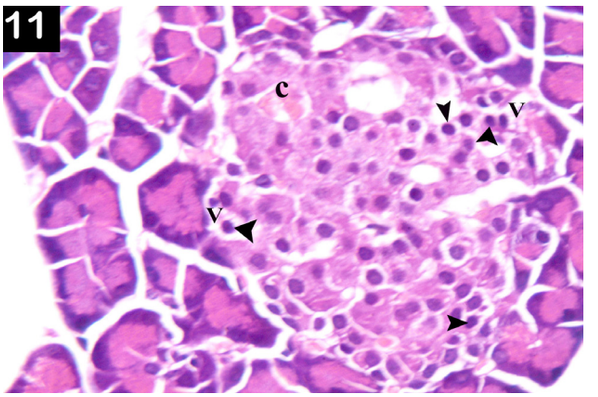


Fig. 11: A photomicrograph of a pancreas from energy drink-treated group showing the islet cells containing multiple variable sized cytoplasmic vacuoles (V) and small darkly stained nuclei (arrow heads). Notice the dilated congested blood capillaries (C) in-between the islet cells. (H&E X 1000)

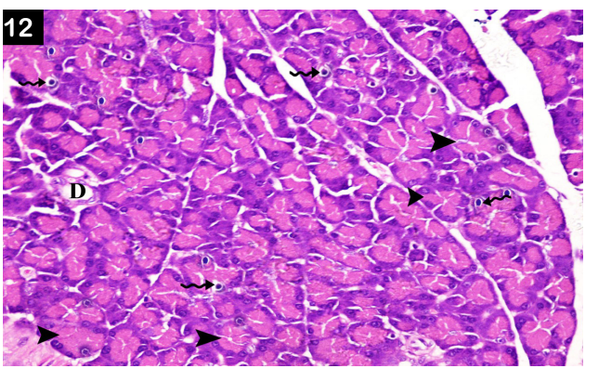


Fig. 12: A photomicrograph of a pancreas from avocado oil-energy drink treated group showing few pancreatic acinar cells containing darkly stained nuclei with perinuclear halo (wavy arrows) between nearly normal pancreatic acini (arrow head). Notice the normal pancreatic duct (D) (H&E X 400)

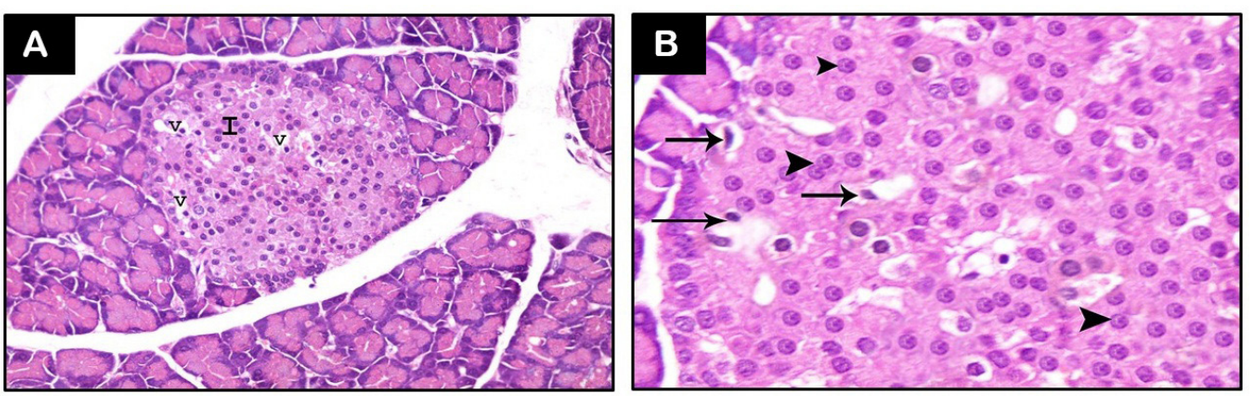


Fig. 13: A photomicrograph of a pancreas from avocado oil-energy drink treated group. (A): showing an islet of Langerhans (I) with preserved normal architecture. Some islet cells appear with vacuolated cytoplasm (V). (B): showing an islet of Langerhans having normal cells having pale stained nuclei with prominent nucleoli (arrow heads). Few vacuolated islet cells are seen having darkly stained nuclei (thin arrows). (H&E, (A): X 400, (B): X 1000)

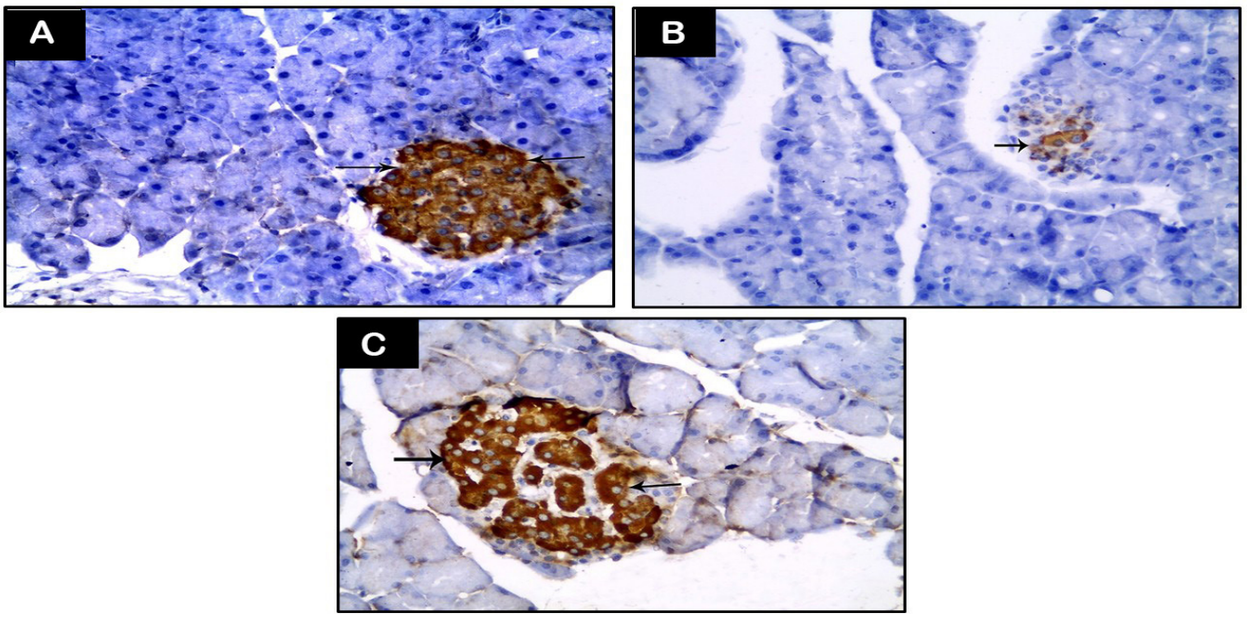


Fig. 14: A photomicrograph of a pancreas with insulin immunostaining. (A): control group showing a strong positive cytoplasmic reaction of β cells (arrows). (B): energy drink-treated group showing a weak positive cytoplasmic reaction of few β cells (arrow). (C): avocado oil-energy drink treated group showing a strong positive cytoplasmic reaction of β cells (arrows). (Insulin immunostaining X 400)

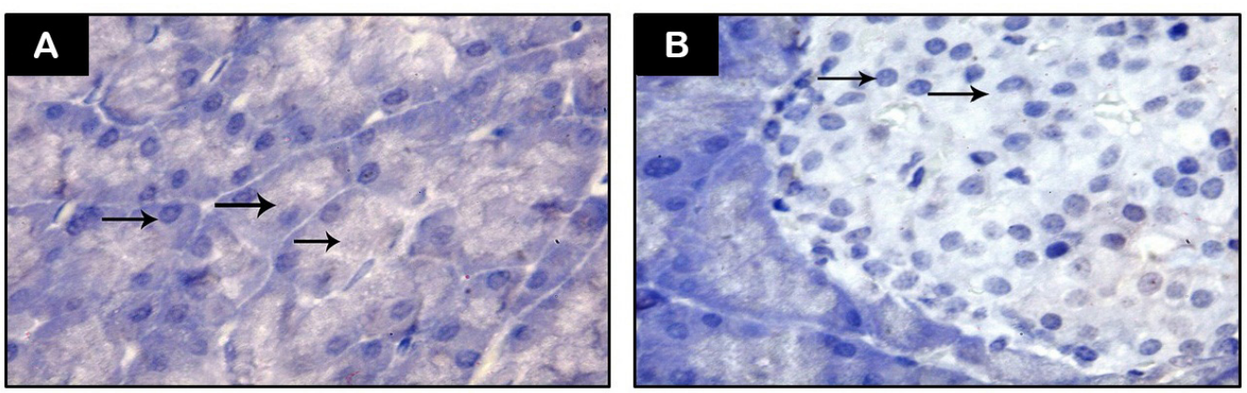


Fig. 15: A photomicrograph of a pancreas with active caspase-3 immunostaining from control group. (A): showing negative caspase-3 reaction in pancreatic exocrine acinar cells (arrows). (B): showing negative caspase-3 reaction in the cells of Islet of Langerhans (arrows). (Active caspase- 3 immunostaining X 1000)

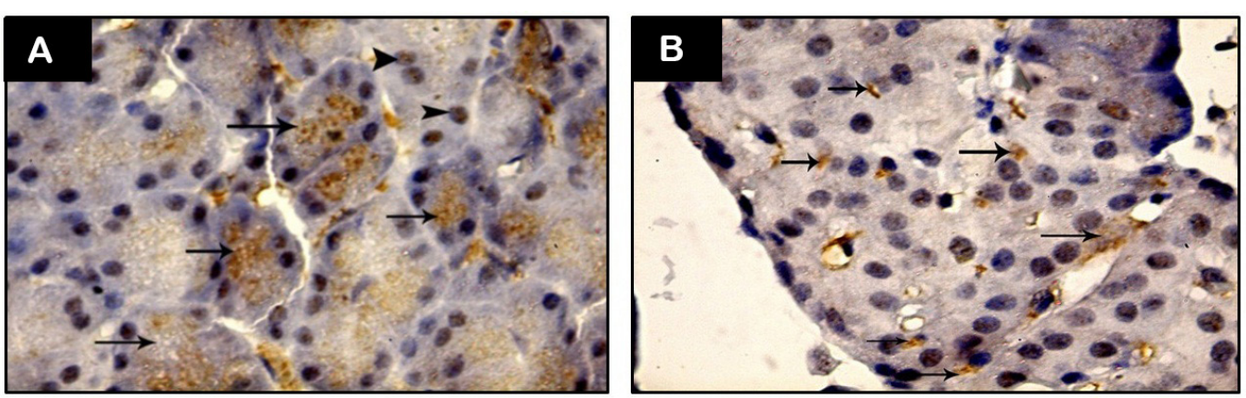


Fig. 16: A photomicrograph of a pancreas with active caspase-3 immunostaining from energy drink treated group. (A): showing a strong positive cytoplasmic (arrows) and nuclear (arrow heads) caspase-3 reaction in many pancreatic acinar cells. (B): showing a strong positive cytoplasmic caspase-3 reaction in many cells of an islet of Langerhans (arrows). (Active caspase- 3 immunostaining X 1000)

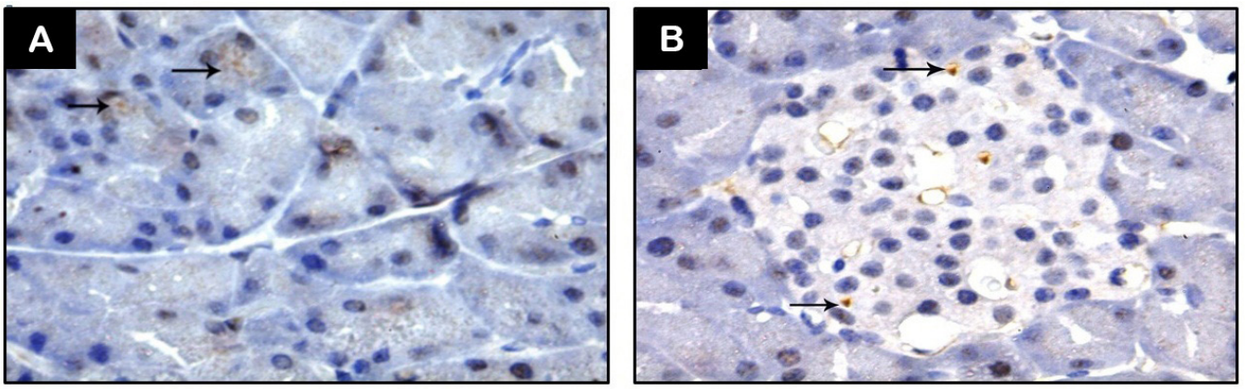


Fig. 17: A photomicrograph of a pancreas with active caspase-3 immunostaining from avocado oil-energy drink treated group. (A): showing a weak cytoplasmic caspase-3 reaction in few acinar cells (arrows). (B): showing weak cytoplasmic caspase-3 reaction in few cells of an islet of Langerhans (arrows). (Active caspase- 3 immunostaining X 1000)

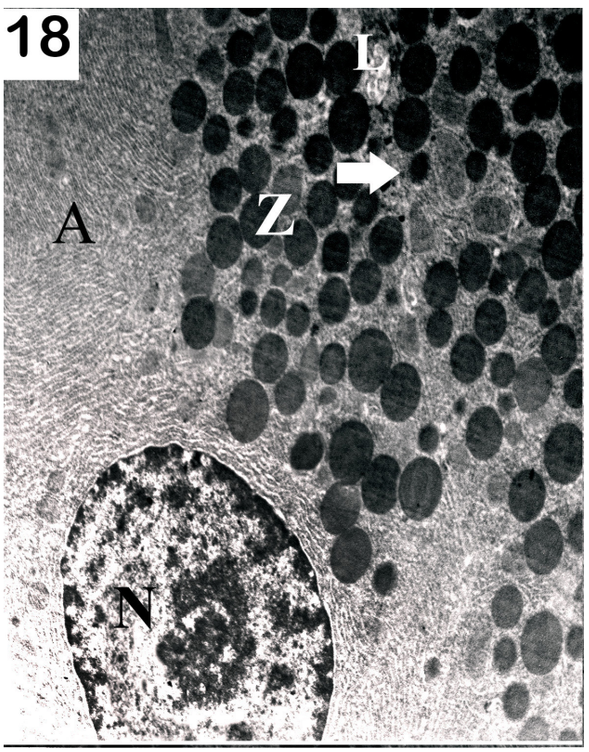


Fig. 18: An electron micrograph of a pancreas of control group showing part of pancreatic acini with a narrow lumen (L) containing the apical microvilli of the acinar cells. The acinar cell (A) has a basal rounded euchromatic nucleus (N) and an apical numerous electron dense zymogen granules (Z). A narrow intercellular space (thick short arrow) is seen between the acinar cells. (Mic. Mag. X 2000)

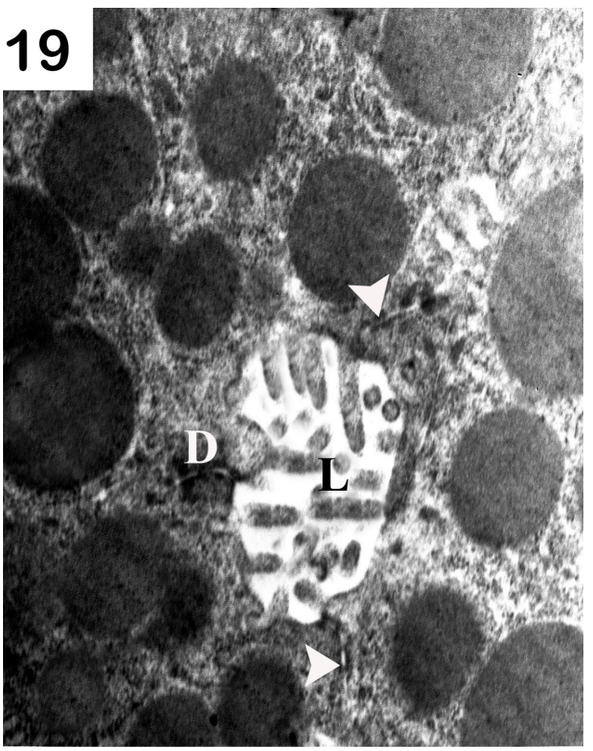


Fig. 19: An electron micrograph of a pancreas of control group showing an acinus with normal lumen (L) containing multiple microvilli. Narrow intercellular spaces (arrow heads) and desmosomal junction (D) can be seen in-between the lateral wall of the acinar cells. (Mic. Mag. X 8000)

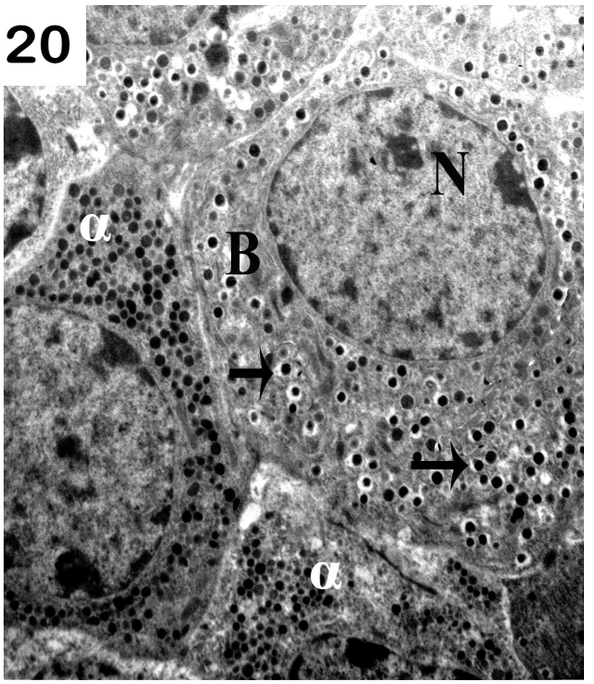


Fig. 20: An electron micrograph of a pancreas of control group showing β cell (B) having rounded euchromatic nucleus (N) with regular contour. The cytoplasm contains numerous electron dense granules (arrows). Notice parts of alpha cells (α) can be seen containing homogenous electron dense granules. (Mic. Mag. X 2000)

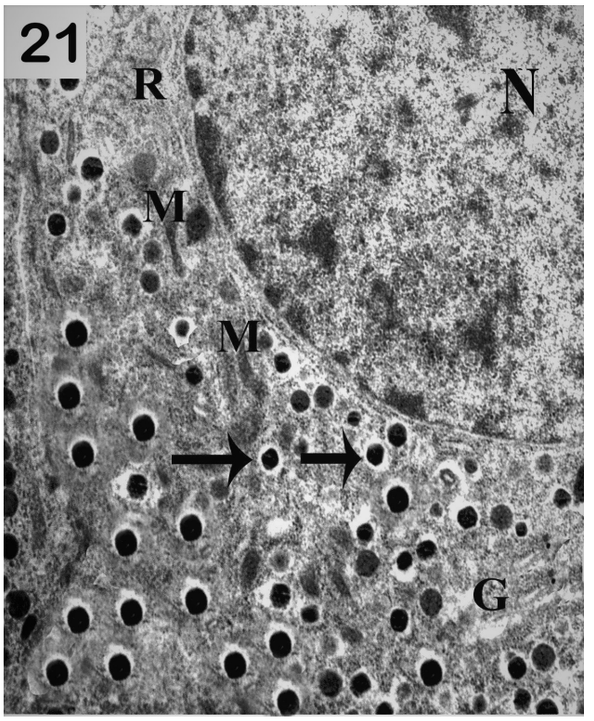


Fig. 21: An electron micrograph of a pancreas of control group showing β cell with part of its nucleus (N). The cytoplasm contains numerous electron dense granules surrounded by wide electron lucent haloes (arrows), multiple mitochondria (M), RER (R) and Golgi apparatus (G). (Mic. Mag. X 4000)

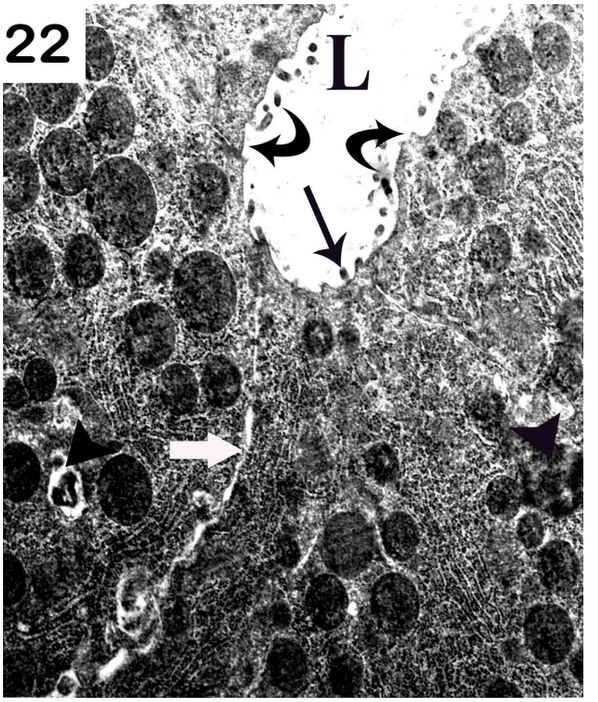


Fig. 22: An electron micrograph of a pancreas of energy drink-treated group showing marked widening of the acinar lumen (L). Notice the shortening of the apical microvilli (arrow) of some acinar cells and absence of them in other cells (curved arrows). Secondary lysosomes are seen in the cytoplasm (arrow heads). Notice wide intercellular space (thick short arrow). (Mic. Mag. X 4000)

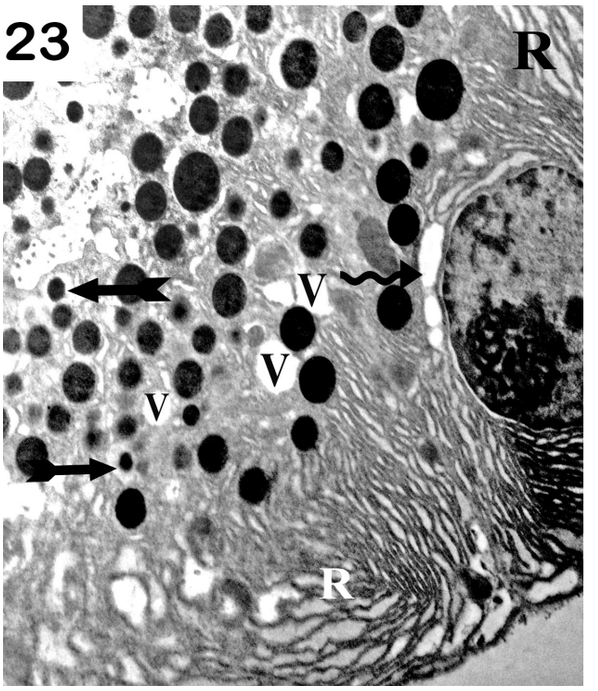


Fig. 23: An electron micrograph of a pancreas of energy drink-treated group showing acinar cells containing cytoplasmic vacuoles (V), peripheral dissolution of zymogen granules (bifid arrows) and dilated RER cisternae (R). Notice the dilated perinuclear cisternae (wavy arrow). (Mic. Mag. X 2000)

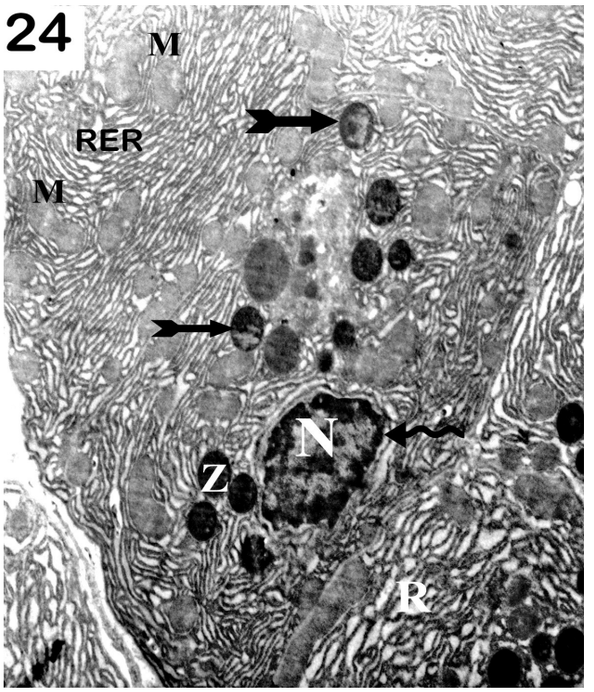


Fig. 24: An electron micrograph of a pancreas of energy drink-treated group showing loss of normal architecture of pancreatic acinar cell, the acinar cell has a shrunken irregular nucleus (N) with condensed chromatin, swollen mitochondria (M), dilated RER (R) and perinuclear cisternae (wavy arrow). The zymogen granules are few and having heterogeneous content (bifid arrows). Notice the basal migration of some zymogen granules (Z). (Mic. Mag. X 2000)

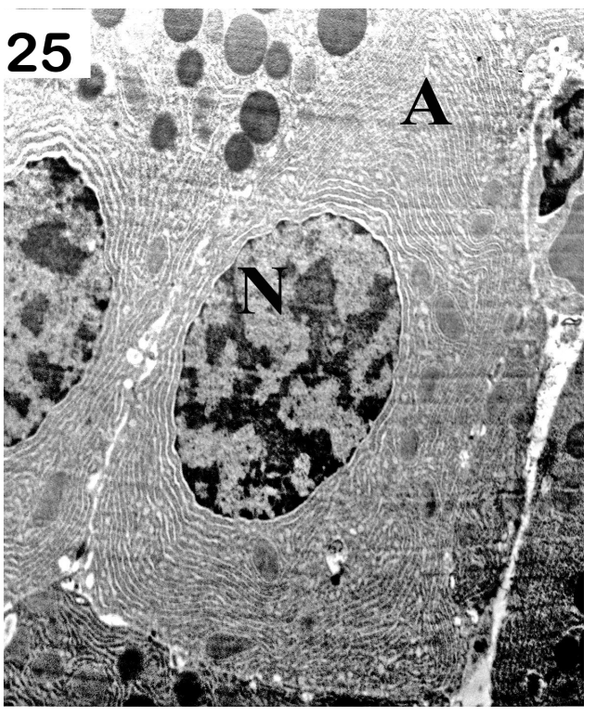


Fig. 25: An electron micrograph of a pancreas of group C showing a pancreatic acinar cell (A) having a nucleus (N) with condensed chromatin with absence of cytoplasmic zymogen granules. (Mic. Mag. X 2000)

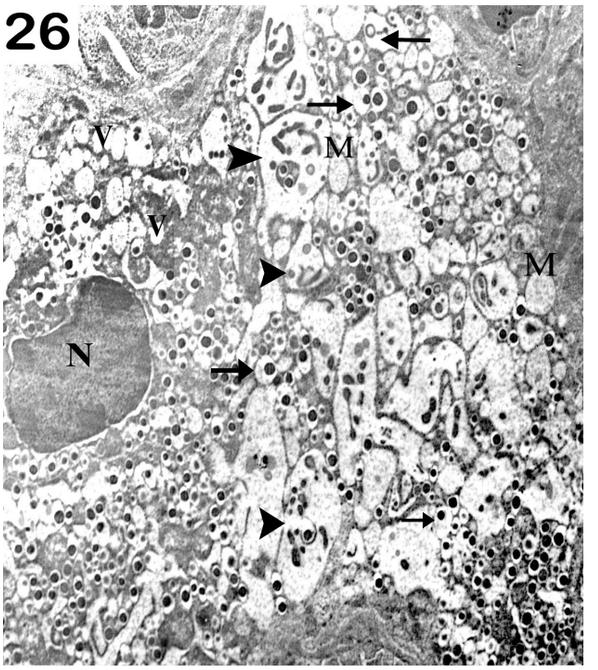


Fig. 26: An electron micrograph of a pancreas of energy drink-treated group showing β cell of the islets of Langerhans have shrunken irregular nucleus (N) with condensed chromatin. The cytoplasm contains electron dense granules surrounded with wide electron lucent halos (arrows). Variable sized cytoplasmic vacuoles (V), swollen mitochondria with destroyed cristae (M) and many autophagic vacuoles (arrow heads) are seen. (Mic. Mag. X 2000)

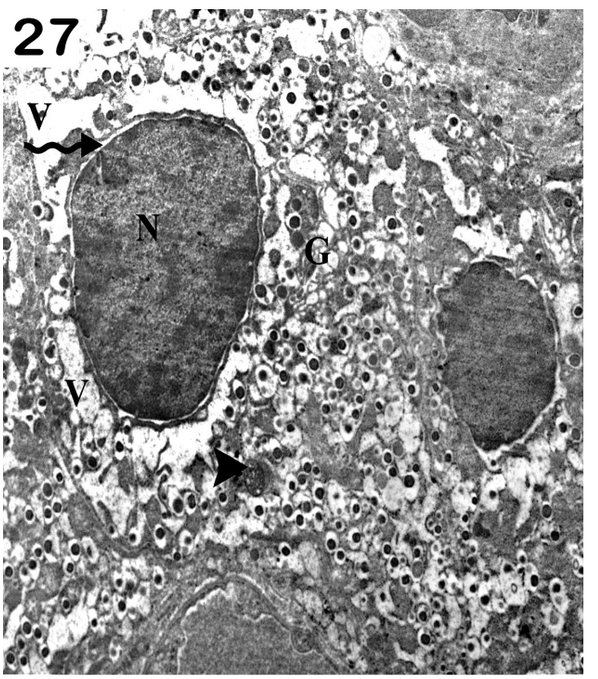


Fig. 27: An electron micrograph of a pancreas of energy drink-treated group showing β cell of the islets of Langerhans having shrunken irregular nucleus (N) with condensed chromatin and dilated perinuclear space (wavy arrow). The cytoplasm contains cytoplasmic vacuoles (V), secondary lysosome (arrow head) and dilated Golgi apparatus (G). (Mic. Mag. X 2000)

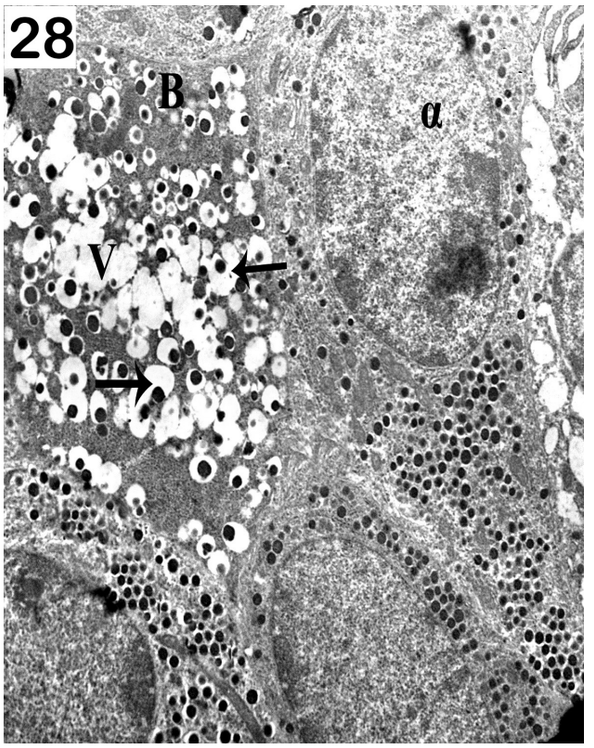


Fig. 28: An electron micrograph of pancreas of energy drink-treated group showing the cytoplasm of β cells (B) containing wide halo spaces around the cytoplasmic granules (arrows) and multiple cytoplasmic vacuoles (V). Notice apart of alpha cell (α) appears containing homogenous and electron dense granules. (Mic. Mag. X 4000)

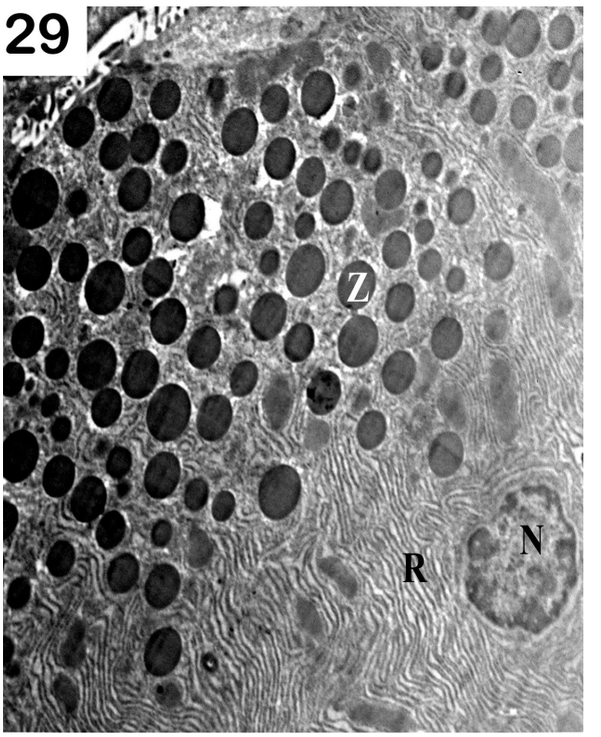


Fig. 29: An electron micrograph of a pancreas of avocado oil-energy drink treated group showing an acinar cell with basal shrunken nucleus (N). The cytoplasm contains relatively mild dilated RER(R) and numerous apically located zymogen granules (Z). (Mic. Mag. X 2000)

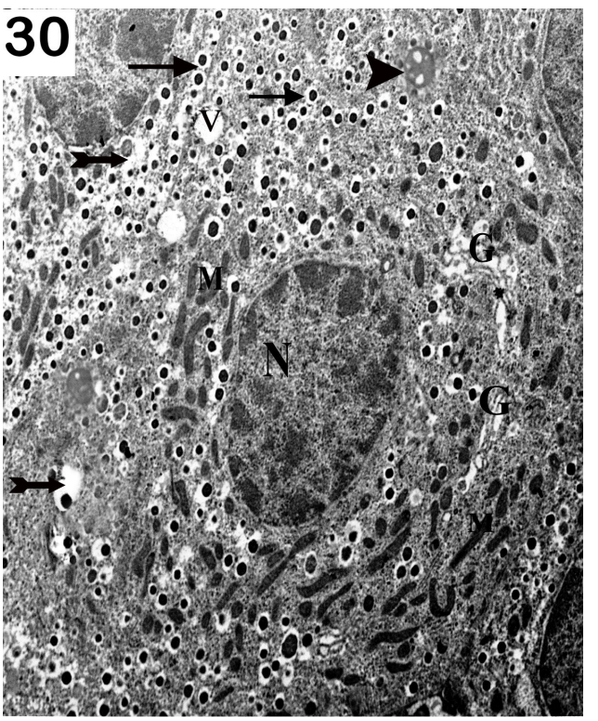
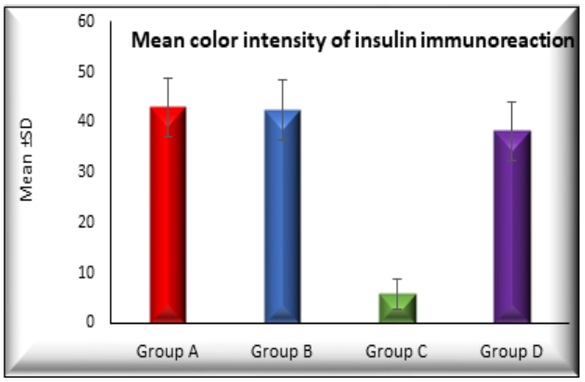


Fig. 30: An electron micrograph of a pancreas of avocado oil-energy drink treated group showing the β cell of islets of Langerhans with relatively normal oval nucleus (N). The cytoplasm contains many mitochondria (M) and numerous cytoplasmic electron dense granules (arrow) but few of them appear with wide halo space around their cores (bifid arrows). Few cytoplasmic vacuoles (V), dilated Golgi (G). Note: A secondary lysosomes (arrow head) is seen. (Mic. Mag. X 2000)

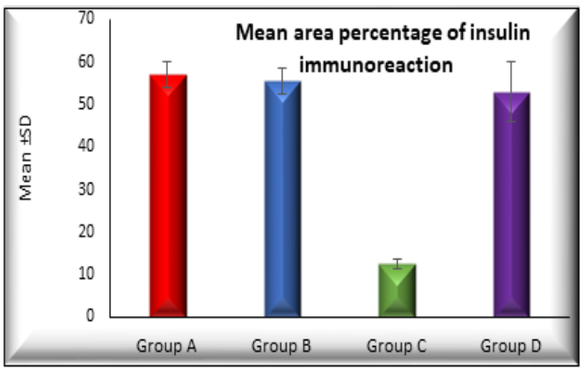
Table 1: Morphometric analysis of the pancreatic specimens in all groups

Groups Parameters	Group A	Group B	Group C	Group D
Mean color intensity of insulin immunoreaction	43.00±5.77	42.57±5.98	5.82±3.01**	38.23±5.74
Mean area percentage of insulin immunoreaction	56.98±2.92	55.35±3.06	12.47±1.03**	52.98±6.88
Mean color intensity of active caspase-3 immunoreaction	1.58±0.97	1.29±0.88	63.64±15.77**	2.08±0.51
Mean number of zymogen granules	50.00±6.70	49.00±5.77	17.20±7.52**	45.50±6.37

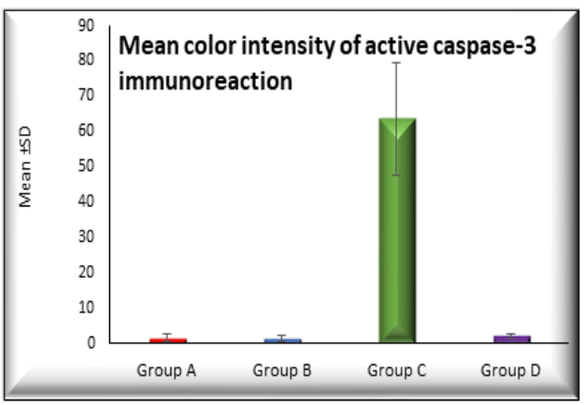
Data is expressed as mean ± standard deviation. ** indicates highly significant vs control.



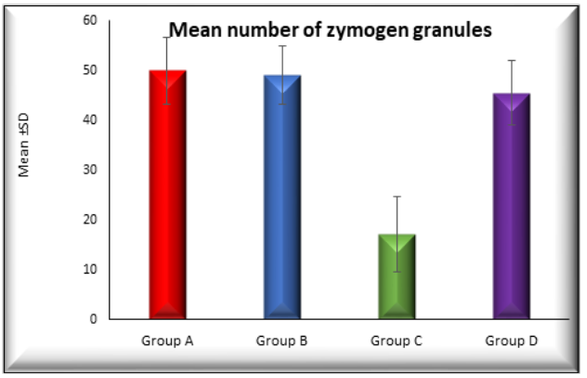
Histogram 1: The mean color intensity of insulin immunoreaction in the different groups



Histogram 2: The mean area percentage of insulin immunoreaction in the different groups



Histogram 3: The mean color intensity of active caspase-3 immunoreaction in the different groups



Histogram 4: The mean number of zymogen granules in different studied groups

DISCUSSION

In the present study, energy drink administration caused evident structural changes in the exocrine and endocrine pancreatic tissues. Cytoplasmic vacuoles and shrunken darkly stained (pyknotic) nuclei were observed in the acinar cells and cells of islets of Langerhans. Furthermore, dilated congested blood vessels were observed in both exocrine and endocrine tissues of pancreas. Moreover, some ducts appeared dilated with retained secretion in addition to mononuclear cellular infiltration. These results were similar to that reported by some researchers[17,18]who attributed these structural changes to the inflammatory response evoked by energy drinks with subsequent release of pro-inflammatory cytokines that cause multiple degenerative changes.

Many studies showed that most of energy drinks negative effects were caused by the active ingredients present in them especially caffeine that produces imbalance of oxidant–antioxidant environment with increased oxidant stress in tissues due to the increased production of inducible nitric oxide synthase (iNOS) and tumor necrosis factor-alpha (TNF-α) that causes cell damage[19,20,21]. In addition, some investigators[22,23] discovered that caffeine decreases the tissue sensitivity to insulin, impairs glucose metabolism and increases stress hormone secretion, and these effects lead to an increase in blood glucose level, lipolysis, glucogenesis associated with decreased glucose consumption peripherally through inhibition of glycolytic enzymes, and this hyperglycemic state leads to glycation of membrane phospholipids of the cell membrane or

cytoplasmic organelles leading to lipid peroxidation and DNA damage in different organs.

In the present study, the cytoplasmic vacuolations of acinar and islet cells that were observed in energy drink treated group could be attributed to fatty degeneration and accumulation of degenerative materials within the cytoplasm[24]. In addition, these vacuolations might be a result of the energy drink induced oxidative stress that damages the cell membrane as well as the membranes of organelles leading to an increase in their permeability and failure of energy-dependent Na+ K+ ion pumps with subsequent accumulation of Na+ inside the cells with entry of water resulting in cellular swelling and vacuolations[25].

In addition, some acinar cells of energy drink treated group were replaced with homogenous acidophilic substance giving the cell a glassy or homogenous appearance. Some authors[26] attributed this to be a feature of necrosis caused by oxidative stress leading to leakage of enzymes with subsequent cellular destruction, degradation of cytoplasmic contents followed by rupture of cells membrane with releasing of internal contents outside the cells.

Furthermore, dilated pancreatic ducts with retained secretion were detected in this study, and this was aligned with the results of other researchers[27,28] who contributed these changes to the accumulation of secretion resulting from the pancreatic tissue injury and dysfunction. In addition, other authors[29,30] explained these changes to be due to the oxidative stress that leads to mitochondrial damage with decreased adenosine triphosphate (ATP) production and decreased energy needed by the duct cells to transport secretion leading to duct dilatation.

The present study revealed congested dilated blood vessels with extravasation of erythrocytes in between the acini, this was in agreement with the results of some authors[31,32] who attributed these changes to the microcirculatory disturbances caused by excess production of nitric oxide resulting from the oxidative stress condition. This leads to increased fragility of the blood vessels walls making them susceptible to produce hemorrhage within the pancreatic tissue and endothelial dysfunction in the form of endothelial cell detachment of blood vessels wall. In contrast, others[33] had reported that excessive drinking of energy drinks could produce myocardial ischemia through vasospasm in coronaries due to different reaction of taurine with caffeine in energy drinks. Additionally, it was reported that consumption of energy drinks had been associated with cardiovascular disorders as well as impaired endothelial function[34].

The mononuclear cellular infiltration that was detected in the present study might be due to the inhibitory effect of caffeine on adenosine A2A receptors leading to development of interstitial inflammation[35]. This inflammatory process might explain the induction of acute pancreatitis by energy drinks[36,37]. On the other hand, other researchers[38] explained the mononuclear cellular infiltration to be as an inflammatory response to eliminate a pathologic insult and to remove injured tissue components to allow tissue repair.

In this work, the pancreas of the energy drink treated groups revealed an apparent thickening of the connective tissue septae, and this might indicate a development of pancreatic fibrosis. Actually, the exact mechanisms of the development of pancreatic fibrosis are still unknown. However, this could be attributed to the toxic effect of caffeine as reported by some authors who studied the effect of caffeine on wound healing of rat gingiva and denoted an increased fibrin deposition on the underlying connective tissue[39]. Other investigators contributed pancreatic fibrosis to the release of free radicals, and they added that the pancreatic stellate cells (PSC) play an important role in the development of the pancreatic fibrosis, accumulating evidence from both in *vivo* and in*vitro* studies[40]. However, the exact role of the free radicals in the activation of the pancreatic stellate cells had not been fully elucidated.

The nuclear pyknosis of pancreatic acinar and islet cells was considered as a sign of apoptosis[41]. Similar changes were observed by some authors[42] who confirmed the nuclear degenerative changes by electrophoresis that revealed DNA fragmentation. The DNA damage or shrinkage and clumping of the nuclear chromatin is the feature of apoptosis[43]. These nuclear changes were confirmed by the EM examination of pancreatic acinar and islet cells, and this might be due to the alteration of actin cytoskeletal structure of such cells caused by caffeine administration leading to apoptosis[44]. Furthermore, these results were confirmed by the immunohistochemical results of this work that revealed a significant increase in the mean color intensity of active caspase-3 immunoreaction in the cytoplasm of most pancreatic acinar cells and some islets cells. Active caspase-3 antibodies are considered as biomarker for apoptosis[45].

Ultrastructurally, the electron microscopic findings confirmed the previous light microscopic ones at the level of cellular organelles. Cytoplasmic vacuolations and dilatation of RER as well as perinuclear cisternae were detected in this study, and these are features of acute cell swelling that occur secondary to loss of control of water uptake as energy drinks interfere with the regulatory mechanisms of cell volume by disturbing cell membrane permeability, causing injury to cell membrane and depletion of ATP production[46].

The mitochondrial swelling observed in the present study might be due to water and solutes entry into the mitochondrial matrix following mitochondrial injury resulting from the oxidative stress induced by caffeine administration[26].

The EM results revealed presence of secondary lysosomes with heterogeneous contents in the cytoplasm of acinar cells. The release of ROS as well as the occurrence of lipid peroxidation requires the presence of lysosomes and activation of lysosomal hydrolytic enzymes as reported by some authors[47,48]. In addition, others[49,50] attributed these

findings to the increased autophagy enhanced by caffeine leading to increased autophagic vacuoles in the cytoplasm.

As regards the zymogen granules of the pancreatic acinar cells, the EM results revealed decreased number of zymogen granules in energy drink treated group, and this was supported by the morphometric results of the present study that showed statistically highly significant decrease in the mean number of zymogen granules in energy drink treated group as compared with control group. This was in agreement with some authors[51,52] who attributed this to the increase in cytosolic calcium which passes rapidly to the nucleus leading to an increase in nuclear calcium which may inhibit the secretory function of pancreatic acinar cells and causes decreased number of zymogen granules.

In addition, the peripheral dissolution of zymogen granules that was observed in the present study might be attributed to the defect in synthesis of zymogen granules sub-membranous matrix which is involved in zymogen granule formation in pancreatic acinar cells. This matrix allows adhesion of the granular content to its surrounding membrane[53]. Moreover, the change in density of zymogen granules might be a result of different degrees of maturation of secretory products and increased activity of zymogen granules[54,55].

In the results of the present study, β-cells of islets of Langerhans were the mostly affected cells in energy drink treated group. The EM results revealed the ultrastructural changes of β-cells that appeared having small electron dense cores with increased electron lucent halos around them. The immunohistochemical results of the present study confirmed the effect of energy drink on B cells of islets of Langerhans as they showed a highly significant decrease in the mean color intensity of insulin immunoreaction in energy drink treated group as compared to control group. This might be due to over stimulation of insulin-secreting cells in response to energy drink administration leading to increased insulin resistance[56]. Moreover, this change might be due to decreased insulin synthesis as some investigators had reported that the degree of granulation of endocrine β-cells depends on their secretion of insulin[57]. Also, it was reported that β-cell degranulation is usually due to depletion of secretory stores of insulin in the damaged cells caused by oxidative stress[58].

In addition, widening of the intercellular spaces was an EM finding in the present work. This coincided with some authors[43] who stated that when fluid leaks into the intercellular spaces, isolation of cells from each other occurs. During cell injury and the acute swelling process, breaking down of cellular junctions occurs secondary to disruption of the cytoskeletal filaments as well as suppression of production and disintegration of the junctional adhesion molecules with consequent separation of the neighboring cells from each other. In addition, it was reported that the oxidative stress caused by caffeine administration also leads to the cytoskeletal changes and this can explain the shortening and absence of acinar cells microvilli that were observed in the present study[59,60].

At the protective level, the co-administration of avocado oil with the energy drink illustrated evident improvement in the structural changes of the exocrine and endocrine pancreatic tissue induced by the energy drink. The protective effect of avocado oil was attributed to its antioxidant effect as documented by some authors who proved that avocado oil can protect phospholipids of cell membrane against oxidative damage by scavenging free radicles at the lipophilic core of the membranes[61]. The avocado oil anti-lipoperoxidative activity is due to accumulation of antioxidants present in avocado oil in the mitochondrial membranes increasing their resistance to lipid peroxidation. Moreover, the avocado oil contains some carotenoids with antioxidant properties including lutein, violaxanthin, antheraxanthin and neoxanthin[62], of which lutein has been suggested to make favorable effects in diabetic disease by decreasing ROS production[63,64,65]. Also, carotenoids are associated with cell protection, cell growth and apoptosis regulation[66].

Furthermore, other clinical studies[67] found that consumption of avocado oil leads to an improvement in the postprandial profile of glycemia, insulin, low density lipoproteins, total cholesterol, triglycerides and inflammatory parameters, such as C-reactive protein (CRP) and interleukin 6. Some investigators[68] suggested that dietary addition of 5–20% avocado oil could increase insulin sensitivity and reduce insulin resistance induced by a diet high in sucrose in rats due to the beneficial effect of monounsaturated fatty acids. Also, the monounsaturated fatty acids in avocado oil counteract the inhibitory effect of the inflammatory cytokine TNF-α on insulin production in rat pancreatic beta cell[69].

Furthermore, the administration of avocado oil could decrease the signs of inflammation such as mononuclear cellular infiltration and dilated congested blood vessels. This could be due to the anti-inflammatory effect of avocado oil that was proved by some authors[70] who reported that avocado oil can inhibit the production of pro-inflammatory cytokines essential for prostaglandin production and lipoxygenase enzyme essential for leukotriene production. This anti-inflammatory effect could be attributed to the polyunsaturated fatty acids content in avocado oil[71].

According to the previous results, it could be concluded that energy drinks induced damaging effects on the histological structure of the exocrine and endocrine part (specially B cells) of pancreas of adult male albino rats, and avocado oil administration could to some extent ameliorate these effects. So, avocado oil administration in concomitant with energy drinks is recommended for people who are susceptible to energy drink toxicity.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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الملخص العربى

تأثير مشروب الطاقة على بنكرياس ذكر الجرذ الأبيض البالغ والدور الوقائى المحتمل لزيت الأفوكادو. دراسة هستولوجية وهستوكيميائية مناعية

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مقدمة: تعد مشروبات الطاقة من المشروبات المنتشرة علي نطاق واسع والتي توجه للشباب كمنشطات لزيادة التركيز الذهني. وأن تناولها يؤدى إلى العديد من المشاكل الصحية. زيت الأفوكادو هو زيت طبيعي مستخلص من فاكهة الأفوكادو و لديه تأثيرات مضادة للأكسدة ومضادة للإلتهابات.

الهدف من البحث: أجرى هذا العمل لدراسة تأثير مشروب الطاقة على التركيب الهستولوجى للبنكرياس فى ذكر الجرذ الأبيض البالغ وتقييم الدور الوقائي المحتمل لزيت الأفوكادو.

المواد وطرق البحث: تم تقسيم ٤٠ من ذكور الجرذان البيضاء البالغة إلى أربع مجموعات؛ المجموعة الضابطة, مجموعة زيت الأفوكادو ٠,٤) ملي /١٠٠ جم), مجموعة مشروب الطاقة (١,٥ملي/١٠٠ جم) و مجموعة زيت الأفوكادو ومشروب الطاقة. و تم اعطاء كل ا الجرذان الجرعات عن طريق الفم مره واحده في اليوم لمدة أربعة أسابيع متواصلة ثم تم تجهيز عينات للفحص بالميكروسكوب الضوئي والإلكتروني. ولقد أجريت دراسة هستوكيميائية مناعية باستخدام أجسام مضاده للأنسولين وcaspase-٣.

النتائج: أظهرت مجموعة مشروب الطاقة عدم انتظام موضعي في تركيب الجزء الإفرازي والصمي للبنكرياس. ولقد أظهر الجزء الإفرازي للبنكرياس وجود فجوات سيتوبلازمية، أنوية داكنة لخلايا العنيبات بالإضافة إلي أوعية دموية محتقنة. وقد أظهر التركيب الدقيق لخلايا العنيبات وجزر لانجرهانز وجود فجوات سيتوبلازمية وتورم الميتوكوندريا مع تحطم الأعراف وتوسع في الشبكة الإندوبلازمية الخشنة وجهاز جولجي بالإضافة إلي وجود أنويه داكنة منكمشة مع إتساع الفراغ حول النواة. وأظهرت الدراسة الهستوكيميائية المناعية انخفاضا ذو دلالة إحصائية في التفاعل الهستوكيميائي المناعي للإنسولين في خلايا بيتا مع وجود زيادة ذات دلالة إحصائية في التفاعل الهستوكيميائي المناعي لـ caspase-٣ في خلايا العنيبات وخلايا الجزر. وعلي النقيض قد لوحظت تغيرات بسيطة في الفئران التي تمت معالجتها بزيت الأفوكادو مصاحبا بمشروب الطاقة مع وجود تغيرات غير ملحوظه في التفاعل الهستوكيميائي المناعي.

الإستنتاج: مشروب الطاقة تسبب في حدوث تغيرات تركيبية في بنكرياس الجرذان وأن اعطاء زيت الأفوكادو مع مشروب الطاقة قد استطاع إلي حد ما تخفيف هذه التغيرات.