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Therapeutic and Protective Effect of Wheat Germ Oil on L-arginine Induced Acute Pancreatitis in Adult Albino Rats

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Abstract

Background: Acute pancreatitis (AP) is a disease that still has high morbidity and mortality rates. Oxygen free radicals and cytokines have been mentioned to play a role in the development of L-arginine induced AP. There is no specific therapy for AP. Herbals, as botanical medical treatments, have generated a deal of public controversy in recent years. Wheat germ oil (WGO) is one of these treatments that possess anti-inflammatory properties.

Objective: To investigate the possible protective and therapeutic effect of wheat germ oil on L-arginine induced AP in adult albino rats.

Methods: Forty adult male albino rats were divided equally into five groups: Group I (control group). Group II (WGO group): received WGO by oral gavage once daily for 3 successive days. Group III (AP group): received double IP injection of L-arginine with 1 h interval. Group IV (protection group): received WGO as group II, followed by L-arginine injection as group III. Group V (therapeutic group): received L-arginine followed by WGO. Blood samples were taken for biochemical assessment of serum lipase to confirm induction of AP. At the end of the experiment, animals were sacrificed and specimens from head of the pancreas were taken and prepared for light, electron microscopic examinations and ELISA detection of interleukin-1β.

Results: AP and protected groups showed extensive acinar cell damage in the form of loss of basal basophilia, necrotic changes, interstitial edema, severe inflammatory infiltration and hemorrhage. These groups showed statistical significant increase in the mean of pancreatic damage compared to control group (p<0.05). Ultrastructurally, the acinar cells showed dense heterochromatic nuclei with many degraded electron-lucent zymogen granules and swollen, disarranged rER. However, the acini showed significant improvement in the therapeutic group.

Conclusion: WGO has a therapeutic not a protective effect on L-arginine-induced AP which might be through its anti-inflammatory effects.

Keywords: L-arginine-induced acute pancreatitis; Serum lipase; Wheat germ oil; Interleukin-1β

Introduction

Acute pancreatitis (AP), an inflammatory disease of the pancreas, is mild and resolves itself without serious complications in 80% of patients; however, in 20% of patients it has complications and a substantial mortality [1]. Worldwide annual incidence of acute pancreatitis is increased about 10-fold over the past 40 years without known reasons [2].

Its etiology and pathogenesis have been intensively investigated for centuries worldwide [3]. In 75%-85% of patients, common bile duct and/or pancreatic duct obstruction by migrating gall stones (38%) and alcohol abuse (36%) are the most frequent causes of AP [4,5]. Duct obstruction promotes pancreatitis by increasing duct pressure and subsequent unregulated activation of digestive enzymes [6]. Moreover, many other etiologies are recorded such as; viral infection (mumps and hepatitis type A&B), drugs (tetracycline, valproic acid, estrogens and furosemide), hypercalcemia, hypertriglyceridemia and developmental abnormalities of the pancreas [7].

Experimental evidence suggests that up-regulation of inflammatory mediators including interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, nitric oxide (NO), adhesion molecules and oxygen free radicals; as pro-inflammatory mediators are excessively produced during AP. These mediators play roles in the intiation and amplification of the cascade of cytokines and in the development of AP from a local to a systemic complicated disease. The cumulative effect of these mediators eventually leads to vascular leakage, hypovolemia, systemic inflammatory response syndrome (SIRS), shock, and organ failure [8,9].

There are no specific therapies for AP. Medical management is aimed at the control of symptoms with anti-inflammatory agents, steroids, and analgesics. As a result of the limitations of conventional therapy, there is a need to explore the potential antioxidant and anti-inflammatory agents available from natural sources, which are cost-

effective and have several advantages than the synthetic compounds in treating pancreatitis [10,11].

Wheat germ oil (WGO) is one of nature's finest super foods that extracted from the germ of the wheat kernel. It is particularly high in policosanol contents, especially octacosanol, which increase the physical performance [12] and can be beneficial natural agents for optimizing cardiovascular functions and improving blood dyslipidemia [13]. At the same time, wheat germ oil is a good source of polyunsaturated fatty acids especially linoleic and linolenic acids (precursors for omega 6 and omega 3 fatty acids) [14] and it is enriched with vitamin E [15]. Previous studies showed that wheat germ oil possesses anti-inflammatory properties [14], decreases oxidative stress [15] and improves lipid metabolism [16]. Also, wheat germ oil increases the regeneration of platelets [17], reduces platelet aggregation, thrombus formation and protects red blood cells membranes from oxidative damage [18].

The aim of this study was to evaluate the prophylactic and therapeutic effectiveness of wheat germ oil in L-arginine induced acute pancreatitis in rats.

Materials and Methods

Animals

In this study 40 adult male albino rats, weighing 200-250 g, were used. All animals received care in compliance with the Guide for Care and Use of Laboratory Animals prepared by Faculty of Medicine, Suez Canal University, Egypt. Animals were housed in clean, properly ventilated stainless steel wire cages under the same environmental conditions. They were maintained on normal rat chow and water ad-libitum. All rats were fasted overnight before each experiment.

Study groups: Animals were divided equally into five groups (eight animals each) and each group was repeated twice:

Group I (control group): Animals of this group was further subdivided into two equal subgroups, subgroup (Ia); animals that didn't receive any medication and subgroup (Ib); animals received normal saline (0.9% NaCl) first as double IP injection of 2.5 ml/kg body weight with 1 h interval then 24 h after the last injection, animals received normal saline for 3 successive days by oral gavage.

Group II (WGO group): Animals of this group received wheat germ oil by oral gavage in a dose of 3 ml/kg body weight every 24 h for 3 successive days [19].

Group III (AP group): Animals of this group received double intraperitoneal (IP) injection of L-arginine in a dose 250 mg/100 g body weight with 1 h interval [20].

Group IV (protection group): Animals of this group received WGO in a dose of 3 ml/kg body weight by oral gavage every 24 h for 3 successive days, then 24 h after the last treatment, animals received double L-arginine injection in the same dose of group III with 1 h interval for induction of AP.

Group V (therapeutic group): Animals of this group received double L-arginine injection in the same dose of group III with 1 h interval for induction of AP, then 24 h after the last injection, animals received WGO in a dose of 3 ml/kg body weight by oral gavage every 24 h for 3 successive days.

Drugs and chemicals

Wheat germ oil: Wheat germ oil was purchased from El-Captain Company (CAP PHARMA), 6th October City, Egypt.

L-arginine: L-arginine powder (extra pure AR 0120, Scharlau Chemie S.A., La Jota, Barcelona, Spain) prepared as a solution by dissolving it in 0.9% saline to a final concentration of 500 mg/ml and the pH was adjusted to 7 with 0.1 M (0.1 N) sodium hydroxide (NaOH) solution.

Biochemical assessment of serum lipase: Two Blood samples were obtained from tail vein of the rats [Group I samples were taken from subgroup (Ib) only]; the first sample was obtained immediately before AP induction (considered as the basal level). The second was obtained 24 hours after AP induction (to confirm the induction of AP in groups III, IV, and V).

Blood samples were centrifuged at 12000 rpm for 10 min and serum was extracted and used for determination of serum lipase level using an autoanalyzer (Hitachi 912 auto-analyzer; Germany).

Histological study

Animals were anaesthetized and perfused with 4% paraformadehyde in 0.1 M sodium phosphate buffer (pH 7.4) 72 hours after induction of pancreatitis [21] (or commencement of study in groups I and II). After perfusion, abdomen of the animals was immediately opened and the pancreas was removed and specimens from the head of pancreas were taken, prepared for light and electron microscopic examinations. ELISA detection for inflammatory marker interleukin-1β was performed as following:

- For light microscopic examination: Specimens were fixed in 10% neutral-buffered formalin solution. Paraffin sections of 5 μm thickness were prepared and stained with H&E and Masson's trichrome stain [22].

- For electron microscopic examination: Specimens (about 1 mm3 in size) were fixed in 2.5% glutaraldehyde solution for 24 hours at 4°C and post-fixed in 2% osmium tetraoxide for one hour, then dehydrated and embedded in resin, from which semi-thin sections were stained with toluidine blue. Ultrathin sections were cut and stained with uranyl acetate and lead citrate [23]. The grids were then examined and photographed using Transmission Electron Microscope (JEOL-EX 1010 TEM) in the Regional Center for Mycology and Biotechnology (RCMB) at Al-Azhar University.

- For ELISA detection of inflammatory marker interleukin-1β (IL-1β): Enzyme-linked immunosorbent assay (ELISA) was used to detect IL-1β Level in pancreatic tissue homogenate. A part of head of pancreas was weighed and homogenized with phosphate buffered saline (PBS, pH 7.4) (1:5 W/V) in an Ultra-Turrax homogenizer (IKA Labortechnik, Staufen, Germany). The homogenate was then centrifuged at 3,000 rpm for 10 min. at 4°C and supernatants were divided into aliquots and kept at -80°C for the assessment of IL-1β. The assay was performed using a commercially available rat IL-1β ELISA kit (Quantikine ®; R&D Systems, Minneapolis MN, USA), according to the manufacturer's instructions. The results were calculated from the standard curve and expressed as pg/ml.

Morphological Analysis

In H&E-stained sections, five non overlapping high-power fields (×400)/sections, and three random sections were examined per animal. Pancreatic damage was assessed and scored using the modification of the grading system described by Schmidt's standards [11] for acinar cell degeneration, interstitial inflammation, edema and hemorrhage. The scales were as following:

- Edema 0: absent or rare; 1: edema in the interlobular space; 2: edema in the intralobular space; 3: isolated-island shape of pancreatic acinus.
- Acinar cell necrosis 0: absent; 1: mild; 2: moderate; 3: severe.
- Inflammation 0: absent; 1: mild; 2: moderate; 3: severe.
- Parenchymal hemorrhage 0: absent; 1: mild; 2: moderate; 3: severe.

Statistical Analysis

Kruskal-Wallis test was used for comparison between groups, followed by multiple Mann-Whitney test as post-hoc test. Comparing biochemical parameter data (baseline and after 24 h. serum lipase) was done using Wilcoxon test. Statistical significance was accepted at P ≤ 0.05.

Results

General observation

During the course of the experiment, no mortality had been observed. Meanwhile, rats of groups III, IV and V were noticed to be lethargic and slow in movement after the 2nd injection but gradually recovered after few hours.

Biochemical results

Serum lipase activity in control and WGO groups reached 0.44 ± 0.1 and 0.39 ± 0.1 U/L respectively (basal level) and there was no statistical significant difference after 24 h (P>0.05).

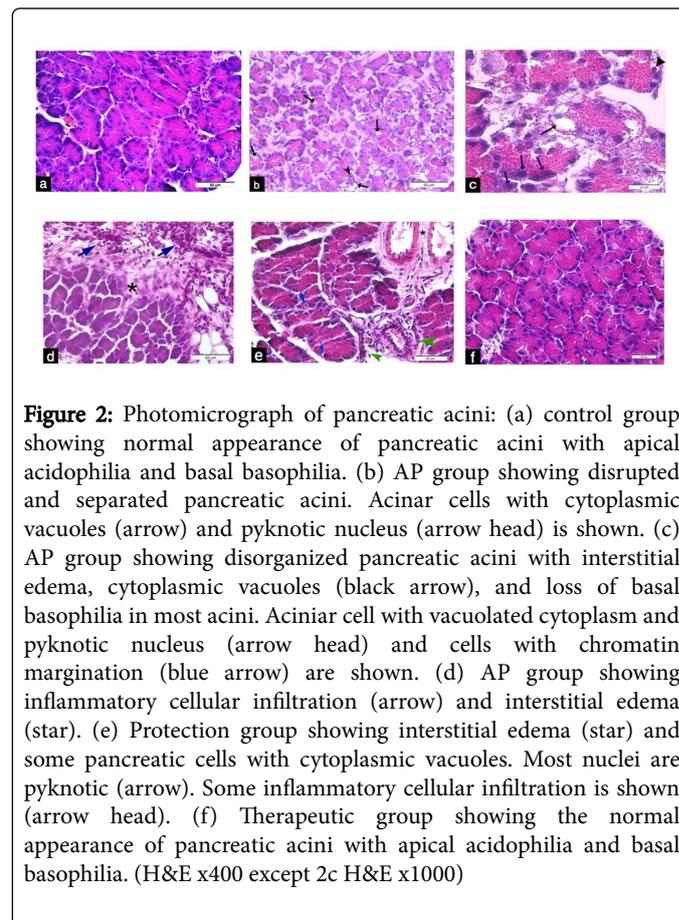**Figure 1:** The serum lipase level (units/L) in different studied groups. Note: **
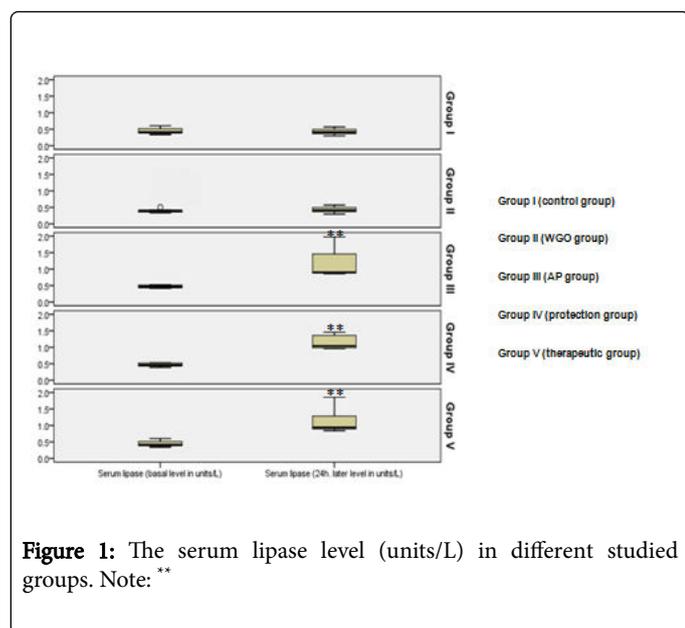
Induction of AP with L-arginine has significantly increased serum lipase activity after 24 h (1.17 ± 0.5 U/L) compared to its basal level (p<0.001). Meanwhile, increased serum lipase activity after 24 h in protection group (1.15 ± 0.2 U/L) and therapeutic group (1.11 ± 0.4 U/L) didn't change by administration of WGO. Paired-t test revealed significant increase in this parameter when compared to the basal level for each group (P<0.05) (Figure 1).

Histological results

The control and WGO groups

- **H&E stained sections** of control subgroups and WGO group showed normal appearance. The pancreatic acini were spherical with closely packed cells and had very narrow acinar lumina. The cells of acini were pyramidal having narrow apical surfaces and broad basal surfaces. Acidophilic zymogen granules appeared in their apical cytoplasm with distinct basophilia in the basal cytoplasm. The basal nuclei were rounded, vesicular with prominent nucleoli (Figure 2a). The score of acinar cell degeneration, interstitial inflammation, edema and hemorrhage was 0 for each of them.

Figure 2: Photomicrograph of pancreatic acini: (a) control group showing normal appearance of pancreatic acini with apical acidophilia and basal basophilia. (b) AP group showing disrupted and separated pancreatic acini. Acinar cells with cytoplasmic vacuoles (arrow) and pyknotic nucleus (arrow head) is shown. (c) AP group showing disorganized pancreatic acini with interstitial edema, cytoplasmic vacuoles (black arrow), and loss of basal basophilia in most acini. Aciniar cell with vacuolated cytoplasm and pyknotic nucleus (arrow head) and cells with chromatin margination (blue arrow) are shown. (d) AP group showing inflammatory cellular infiltration (arrow) and interstitial edema (star). (e) Protection group showing interstitial edema (star) and some pancreatic cells with cytoplasmic vacuoles. Most nuclei are pyknotic (arrow). Some inflammatory cellular infiltration is shown (arrow head). (f) Therapeutic group showing the normal appearance of pancreatic acini with apical acidophilia and basal basophilia. (H&E x400 except 2c H&E x1000)

- **Masson's trichrome stained sections** of both groups showed normal distribution of minimal amount of collagen fibers around pancreatic acini and interlobular ducts (Figure 3a).
- **Ultrastructural examination** of the pancreatic acini of control group as well as WGO group showed closely joined acinar cells with large spherical basal nuclei. Each cell showed abundant

parallel stacks of rough endoplasmic reticulum (rER) with interspersed mitochondria in the basal cytoplasm. Homogeneous electron-dense zymogen granules were concentrated in the apical cytoplasm (Figure 4a).

The acute pancreatitis groups

- **H&E stained sections** of AP group showed extensive tissue damage characterized by disrupted pancreatic acinar architecture. Pancreatic edema was evident causing disorganization and revealed isolated islands shape of the pancreatic acini. The acinar cells showed severe necrotic changes in form of pyknotic, karyorrhectic, and karyolytic nuclei in addition to cytoplasmic vacuolations. Some acinar cells with nuclear chromatin margination were also seen. Most of the acini lost their basal basophilia and the remaining showed less basal basophilia compared to control sections (Figure 2b and 2c).

Score	Control group	WGO group	AP Group	Protection group	Therapeutic group
Overall pancreatic damage	0.00 ± 0.00	0.00 ± 0.00	9.16 ± 0.67a	7.72 ± 0.53a	0.37 ± 0.49c

Table 1: Score for overall pancreatic damage in different groups (mean ± SD). Note: A Significant difference vs. control group, c Significant difference vs. AP group.
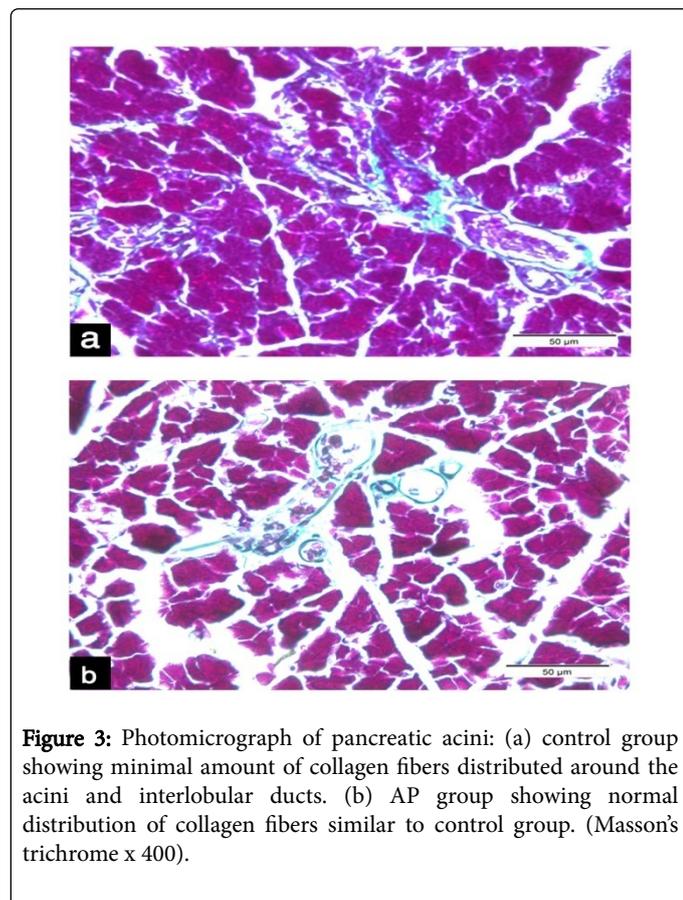
Figure 3: Photomicrograph of pancreatic acini: (a) control group showing minimal amount of collagen fibers distributed around the acini and interlobular ducts. (b) AP group showing normal distribution of collagen fibers similar to control group. (Masson's trichrome x 400).

- Meanwhile, small microscopic foci of hemorrhage and severe inflammatory cellular infiltration were observed mainly in the interlobular space (Figure 2d). No apparent change was observed in cells of the islets of Langerhans. According to Schmidt's standards, pancreatic edema, acinar cell necrosis, inflammation scored 3, while parenchymal hemorrhage scored 1. There was also statistical significant increase in the mean of pancreatic damage compared to control group ($p<0.05$) (Table 1).

- Masson's trichrome stained sections of this group showed no apparent change compared to control group (Figure 3b).

- Ultrastructural examination of the pancreatic acini of AP group showed necrotic changes in acinar cells in the form of large cytoplasmic vacuoles and small dense heterochromatic nuclei. The cytoplasm appeared less crowded with electron-dense zymogen granules while others were electron-lucent and degraded. Rough endoplasmic reticulum (rER) appeared swollen, disarranged and less condensed compared to that of the control group (Figure 4b and 4c).

The protection group

- H&E stained sections of protection group showed approximately similar changes to that of AP group. Diffuse edema was seen mainly in interlobular and intralobular spaces slightly separating the acini from each other. In addition, most of the acini lost their basal basophilia and the acinar cells showed necrotic changes in form of cytoplasmic vacuolations and pyknotic nuclei. Interlobular spaces showed moderate inflammatory cellular infiltration and minimal amount of hemorrhage (Figure 2e). According to Schmidt's standards the pancreatic edema, acinar cell necrosis and inflammation scored 2, while parenchymal hemorrhage scored 1. This group showed statistical significant increase in the mean of pancreatic damage compared to control group ($p<0.05$) however, there was no statistical significant difference compared to AP group ($p>0.05$) (Table 1).

- Masson's trichrome stained sections were apparently similar to control group.

- Ultrastructural examination of the pancreatic acini of protection group showed apparently similar changes to acinar cells of AP group (Figure 4d).

The therapeutic group

- H&E stained sections of therapeutic group showed evidence of improvement of most of histopathological changes seen in the AP group. There was only very few focal areas of acinar cell necrosis and according to Schmidt's standards, the pancreatic edema, acinar cell necrosis, inflammation and parenchymal hemorrhage scored 0 (Figure 2f). There was statistical significant decrease in the mean pancreatic damage compared to AP group ($p<0.05$) and no statistical significant difference compared to control group ($p>0.05$). The mean pancreatic damage score was similar to the control group (Table 1).

- Masson's trichrome stained sections were apparently similar to control group.

- Ultrastructural examination of the pancreatic acini of the therapeutic group was similar to control group (Figure 4e).

Pancreatic IL-1β level

In AP group as well as protection group, pancreatic IL-1β level was significantly elevated (8197.0 ± 802.0 and 2900.7 ± 789.1) when compared to the control group (242.1 ± 28.8) ($P<0.00$). However,

therapeutic group showed significantly reduced pancreatic IL-1β level (395.2 ± 203.1) when compared to AP and protection groups; but it was statistically non-significant when compared to the control group (P=0.98) (Figure 5).
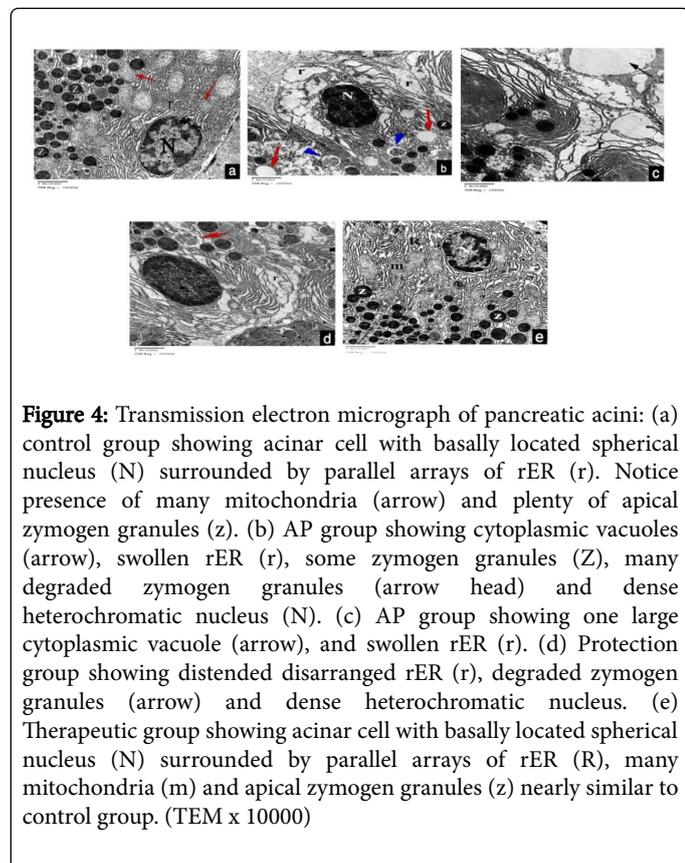
Figure 4: Transmission electron micrograph of pancreatic acini: (a) control group showing acinar cell with basally located spherical nucleus (N) surrounded by parallel arrays of rER (r). Notice presence of many mitochondria (arrow) and plenty of apical zymogen granules (z). (b) AP group showing cytoplasmic vacuoles (arrow), swollen rER (r), some zymogen granules (Z), many degraded zymogen granules (arrow head) and dense heterochromatic nucleus (N). (c) AP group showing one large cytoplasmic vacuole (arrow), and swollen rER (r). (d) Protection group showing distended disarranged rER (r), degraded zymogen granules (arrow) and dense heterochromatic nucleus. (e) Therapeutic group showing acinar cell with basally located spherical nucleus (N) surrounded by parallel arrays of rER (R), many mitochondria (m) and apical zymogen granules (z) nearly similar to control group. (TEM x 10000)
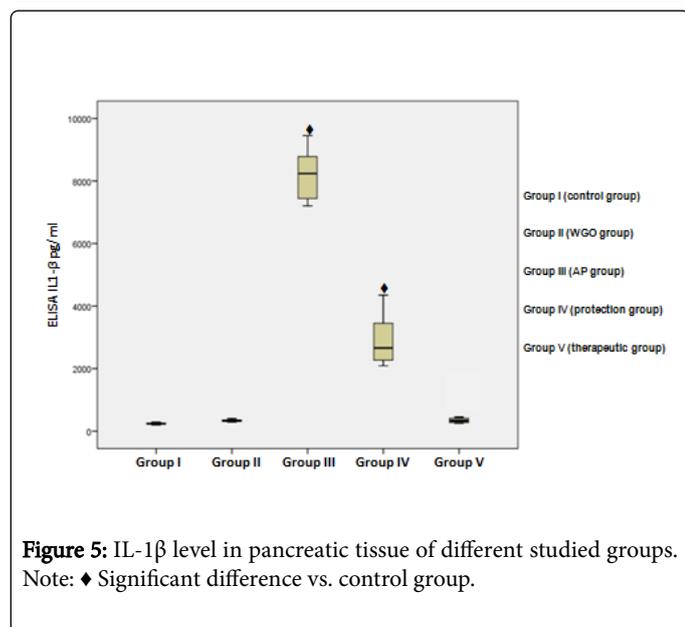
Figure 5: IL-1β level in pancreatic tissue of different studied groups. Note: ♦ Significant difference vs. control group.

Discussion

Acute pancreatitis (AP) is a common cause of emergency hospital admission, with an increase in the incidence rate during the past 30 years. The mortality rates for hospitalized patients vary from 5% to 10% in most series [24]. Current treatment options are limited, and predominantly aimed at supportive therapy [5,25]. This study was designed to assess the possible protective as well as therapeutic effect of Wheat Germ Oil (WGO) in L-arginine induced AP rat model. In this study, intraperitoneal injection of L-arginine was chosen for induction of AP because it is highly reproducible, non-invasive and produces biochemical parameters and histological changes in pancreas resemble that of human situation [26]. Time of decapitation of rats was chosen as revealed in previous work that peak histological changes are observed around 72 h after induction of AP [21]. The results of the present work demonstrate histological and ultra structural alterations in the pancreatic acini of albino rats.

These alterations seem to follow almost the same pattern as that previously observed by other studies used a rat model of L-arginine induced AP [19,26]. In the present work, double IP injection of L-arginine induced severe pancreatic acinar cell necrosis in both AP group and protection group (without any effect on the islets of Langerhans cells) as evidenced biochemically by the increased level of serum lipase 24 hrs after induction of AP and the obvious histological changes that were observed 72 hrs after induction of AP. Serum lipase was affected as it is one of the important diagnostic markers for AP that usually rises within 4-8 h of the initial attack, peaks at 24 h and returns to normal over the 72 h [26-29]. This elevation of serum lipase level could be attributed to activation of pancreatic enzymes within acinar cells and their release into the circulation which followed by marked reduction in pancreatic enzymes production due to extensive pancreatic damage at 72 h [26,30].

In this study, H&E stained pancreatic sections of AP group and protection group showed interstitial edema, cytoplasmic vacuolations with necrotic changes of acinar cells, inflammatory cellular infiltration and microscopic foci of hemorrhage. These histopathological changes observed in the present work were detected and explained by previous researches [10,31,32] who indicated that increased pancreatic levels of reactive oxygen (ROS) and reactive nitrogen species (RNS) contribute to acinar cell injury during pancreatitis, that occur subsequent to intraacinar cell zymogen activation and the release of proinflammatory cytokines [33,34]. The increased levels of pancreatic ROS and RNS lead to membrane lipid peroxidation that damages the membranes of the endothelial cells of the capillaries resulting in increased microvascular permeability that causes significant pancreatic edema [31]. Presence of cytoplasmic vacuolations in acinar cells, observed histologically in this group and with TEM were also observed by Mareninova and colleagues [35]; who found presence of numerous cytoplasmic vacuoles containing nondegraded or partially degraded material (including zymogen granules [ZGs]) in acinar cells in L-arginine induced AP. They suggested that these vacuoles are due to impaired autophagy occurred in AP caused by deficient lysosomal degradation resulted from imbalance between cathepsin L (CatL), which degrades trypsinogen and trypsin, and cathepsin B (CatB), which converts trypsinogen into trypsin, leading to intra-acinar accumulation of active trypsin. Thus, deficient lysosomal degradation may be a dominant mechanism for increased intra-acinar trypsin in AP [34-38]. Another explanation for cytoplasmic vacuoles, may be due to high expression of vacuole membrane protein 1 (VMP1) during L-arginine induced AP which was followed by acinar cell death [39]. In addition to the

increased intra-acinar trypsin level and according to the work of Gukovsky and colleagues [40]; there was a dramatic decrease in pancreatic levels of lysosome-associated membrane protein-2 (LAMP-2) that occur in AP due to defective CatB and also LAMP-2 becomes accessible to lysosomal hydrolases which cause inflammation and acinar cell necrosis that were detected in this study. On the other hand, L-arginine induced AP causes acinar cell mitochondria damage by arginine-induced impairment of ATP synthase [41].

In the present study, the cellular infiltration that observed in pancreas was confirmed by elevated pancreatic IL-1β level in both groups (AP group and protection group). This result comes in concordance with that of Yu-ling and Li-min [41] who reported that serum IL-1β and IL-6 level increased significantly in rats with serious AP induced by large doses of L-arginine injection and they correlated this directly with the histopathological changes. Another study [42] showed that IL-1β caused impaired autophagy via alteration of intracellular Ca^{2+} homeostasis, and impaired autophagy caused accumulation of p62 (an important scaffold in the IL-1β pathway); whose increased expression promotes IL-1β production that aggravate the pancreatic damage that may evolves to a systemic disease. Impaired autophagy contributes to trypsinogen activation and the continuation of this cycle in existing cells will prevent patients with AP from recovering [43]. Once inflammatory infiltration becomes pronounced, and secretion of cytokines from monocytes/macrophages either from the damaged pancreas or from peritoneal macrophage stimulated by IP L-arginine injection begins [44]; the release of primary cytokines as Tumor Necrosis Factor-α (TNF-α) and IL-1β is initiated and amplified the inflammatory cascade in AP by activating mitogen-activated protein kinases (MAPKs) and nuclear factor-κB (NF-κB), which induce the release of chemokines and other cytokines, and a positive feedback loop, which up-regulates their own expression, that leading to deep inflammation [45].

To date, the role of WGO on L-Arginine-induced AP has not been reported, however, we postulated that WGO may have beneficial effects in this disease. WGO is rich in Omega-3 unsaturated fatty acids mainly α-linolenic acid (ALA) which fights inflammation, while protecting the cardiovascular and immune systems [46]. Furthermore, previous studies have shown that linoleic and linolenic acids-rich WGO decreases oxidative stress in patients with mild hypercholesterolemia [15]. This oil is also a source of vitamin E which acts as inhibitor of oxidation processes in body tissues and was shown to protect various biomolecules, such as DNA, membrane lipids and cytosolic proteins from oxidative damage induced by oxygen-derived free radicals [47]. In this study, WGO treatment significantly decreased the histopathological scores of all changes and reversed the elevation of pancreatic IL-1β level in the treated group. Our results showed that WGO attenuated pancreatic tissue injury; this might be through inhibiting the release of inflammatory cytokines IL-1 β. This inhibition could be due to the fact that ALA found in WGO is converted into eicosapentaenoic acid (EPA) and docosahexaenonic acid (DHA) [48]. EPA and DHA were reported to inhibit the production of IL-1β and TNF-α by monocytes [49,50]. On the other hand, in the protected group, in which pancreatitis was preceded by the administration of WGO, we demonstrated that WGO failed in reducing the histopathological changes observed in the pancreatic acinar cells compared to AP group. This failure might be associated with the lack of proinflammatory cytokines secretion in this stage which is necessary for the WGO function.

Conclusions

In conclusion, this study shows that WGO treatment improved the histopathological changes induced by L-arginine in the pancreas. WGO improved these changes when given after induction of pancreatitis but it didn't show any improvement when given before the induction. The mechanism of WGO therapeutic effect may be through its anti-inflammatory effects. However, further studies are warranted to confirm this mechanism.

Acknowledgement

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