Protective Effect of Diclofenac and Enoxaparin in L-Asparaginase Induced Acute Pancreatitis in Rats

Amr El-nashar, Amira M. Abo-Youssef, Ebtehal El-demerdash

ABSTRACT

Objectives: This study was designed to evaluate the protective effect of enoxaparin and diclofenac against L-asparaginase induced pancreatitis. Methods: Acute pancreatitis was induced in rats by intramuscular injection of L-asparaginase (1,000 I.U/Kg) daily for five days. Enoxaparin was given subcutaneous (100 I.U/Kg) and diclofenac was given intraperitoneal (2 mg/Kg) daily for five days. Then, markers of pancreatic injury, lipids, immune cell infiltration and oxidative stress were analyzed with histopathological examination of the pancreatic tissue. Results: During acute pancreatitis, oxidative stress markers were significantly changed as indicated by reduced tissue glutathione and increased malondialdehyde levels. Treatment with either enoxaparin or diclofenac or their combination restored levels of tissue glutathione and induced malondialdehyde levels. This was accompanied by a significant increase in immune cells infiltration as indicated by the high level of myeloperoxidase (MPO) and pro-inflammatory cytokine TNF-alpha. Triglyceride only increased level. Treatment with both enoxaparin or diclofenac or their combination restored biochemical markers including serum alpha-amylase, reduced glutathione, malondialdehyde, pro-inflammatory cytokine TNF-alpha, myeloperoxidase, and triglycerides. Histological injuries of pancreatic tissues as vacuolation and necrosis of epithelial lining pancreatic acini, inflammatory cells infiltration, and focal pancreatic hemorrhage were also reduced by treatment with enoxaparin and/or diclofenac. Conclusions: The present study emphasizes the potential protective effect of enoxaparin and diclofenac against L-asparaginase induced pancreatitis. Keywords: LMWH, MPO, NSAIDs, Pancreas, Triglycerides.

INTRODUCTION

L-asparaginase is a bacterial enzyme that hydrolyzes L-asparagine to aspartic acid and ammonia resulting in depletion of the circulating pool of L-asparagine and inhibition of protein synthesis[1]. Many forms of L-asparaginase enzyme are used in food processing aids and for medical purposes. As a food processing aid, it can reduce up to 90% the amount of acrylamide in starchy foods without changing its taste and color [2]. It is indicated in the clinical treatment protocols for phase III clinical trials of acute lymphoblastic leukemia (ALL) and non-Hodgkin’s lymphoma and phase I/II for other types of cancer as breast, ovarian, brain and pancreatic cancer[3-5]. L-asparaginase is the backbone treatment of ALL in which 6 to 9 doses are required to achieve complete remission status in the induction phase of the treatment protocol and 12 to 19 doses in the maintenance phase [6]. Hypersensitivity reactions with anaphylaxis due to the formation of anti-L-asparaginase antibodies from immunological response were found up to 60% of patients [6, 7]. Liver toxicity indicated by elevated liver enzymes, increased bilirubin, hyperglycemia, and changes in lipid metabolism can be also observed [7].

Acute pancreatitis can also occur during L-asparaginase administration and induces inflammatory response ranging from pre-pancreatitis to direct destruction of pancreatic tissues[8]. Several hypotheses are trying to identify the molecular mechanism of pancreatitis. All of them accepted that the secretory acinar cells (SAC)
have the major role in the pathogenesis of pancreatitis, which contains abnormal active zymogens[9]. Premature activation inside the acinar cell due to internal or external cause induces auto-digestion with massive damage of pancreatic tissue [10]. The high release of reactive oxygen species after stress, with the release of pro-inflammatory cytokine tumor necrosis factor - alpha(TNF-alpha) from infiltrated immune cells and disruption of blood rheology due to increasing blood viscosity or vascular epithelial destruction, are also considered other mechanisms to induce acute pancreatitis[11, 12].

Enoxaparin, low molecular weight heparins(LMWH), is used for the treatment and prophylaxis of thrombosis. Its mechanism of action depends on its ability in inhibition of coagulation factors Xa and IIa which catalyze the conversion of prothrombin to thrombin. Subsequently, it decreases thrombin and prevents fibrin clot formation[13]. Enoxaparin is also co-administrated with antibiotics in the treatment of sepsis[14], chronic disseminated intravascular coagulopathy[15], treatment of arrhythmia, unstable angina and non-Q-wave myocardial infarction[16] and in treatment protocol of human immunodeficiency virus[17]. Enoxaparin shows anti-inflammatory[18], lipid lowering[19], and antioxidant activities[20]. The anti-inflammatory action of enoxaparins is its action on white blood cells (WBCs) by inhibition of the release of proinflammatory cytokines like TNF -alpha, complement activation, and the release of nuclear factor kappa B (NF-kB), Interleukin-8 (IL-8), IL-6 and IL-1b[21].

Diclofenac is a non-steroidal anti-inflammatory drug (NSAID) used for the treatment of pain, hyperthermia, inflammatory disorders, and migraine. It is used in many studies as an investigational drug in the treatment of chronic pain when combined with opioids[22], to enhance mode stabilization when combined with psychoactive drugs[23], and to reduce minimum inhibitory concentration (MIC) when combined with antibiotics [24]. It also shows several activities in many experimental and clinical studies including anti-inflammatory, antioxidant, antiangiogenic, and voltage-dependent calcium channels blocker[25-27]. The anti-inflammatory effect of diclofenac rises from its action on cyclooxygenase (COX) enzymes with two isoforms COX-1 and COX-2 and, therefore, inhibition of the synthesis of prostaglandins and thromboxane from arachidonic acid. Also, it has an inhibitory effect on phospholipase A2 (PLA2), TNF-alpha and L-selectin[28].

This study was designed to evaluate the protective effect of enoxaparin sodium and diclofenac sodium against L-asparaginase-induced pancreatitis, depending on the hypothesis that said acutepancreatitis initiated due to immune cell infiltration which increases the oxidative stress inside the pancreatic tissue. The proinflammatory cytokine TNF-alpha is a key factor in both initiation and propagation of the immune cell infiltration and dissemination of the inflammation. Myeloperoxidase is activated inside the immune cell especially phagocytes and lymphocytes and stimulate the oxidative stress inside the pancreatic tissue. So, by using TNF-alpha inhibitor enoxaparin and NSAID diclofenac, we can protect the pancreatic tissue from damage. Second objective is to evaluate the role of lipid markers as a co-factor in the progression of the disease during enzyme administration.

MATERIALS AND METHODS

Animals:

All the experimental procedures were conducted using male Sprague-Dawley rats weighing (150-200 g) provided by the National Cancer Institute, Cairo, Egypt and left to accommodate in the animal house, for one week before being subjected to experimentation. All animals were maintained under a twelvehourlight–dark cycle, with controlled humidity (60–80%) and constant temperature (22 ± 1°C). Throughout the study, food and water were supplied ad libitum. The study was carried out according to the guidelines of the Ethics Committee, Faculty of Pharmacy, Beni-Suef University.

Drugs, chemicals and reagent kits:

L-asparaginase was purchased from (Medac GmbH, Egypt). Diclofenac Sodium was purchased from (Novartis, Egypt). Enoxaparin sodium was purchased from Sanofi, Egypt, Butanol, potassium hydrogen phosphate (KH2PO4), dipotassium hydrogen phosphate (K2HPO4), trichloroacetic acid (TCA), and formaldehyde 10 % were purchased from El-Gomhoreya Chemical Co, Cairo, Egypt. Ellman's reagent [5,5'-dithio-bis (2-nitrobenzoic acid); DTNP], 1, 1', 3, 3'- tetra methoxy propane, and thiobarbituric acid (TBA) were purchased from Sigma-Aldrich Co., USA. Rat Myeloperoxidase (MPO) ELISA kit was purchased from MyBioSource, San Diego, CA, USA. Rat TNF-alpha tissue lysate ELISA kit was purchased from Ray Biotech, Norcross, GA, USA. Lipase kit was purchased from QuimicaClinicaAplicada S.A, Greece. Kits of alpha-amylase, TC, HDL-cholesterol, LDL-cholesterol, and TG were obtained from Bio-Diagnostic Company (Cairo, Egypt). All other chemicals were obtained from local sources and were of analytical grade.

Experimental Protocol:

Rats were divided into 8 groups, eight animals each; and treated for five days as follows:

Group 1: normal saline 0.9% (this group served as normal control group).
Group 2: L-asparaginase at (1000 IU/kg, i.m.) [29], and this group served as positive control group.
Group 3: enoxaparin sodium subcutaneously (100 IU/Kg) [30]
Group 4: diclofenac sodium intra-peritoneal (2 mg/Kg)[31]
Group 5: enoxaparin sodium and diclofenac sodium
Group 6: L-asparaginase and enoxaparin sodium
Group 7: L-asparaginase and diclofenac sodium
Group 8: L-asparaginase and enoxaparin sodium and diclofenac sodium

At the end of the experimental period, rats were anesthetized and blood samples were collected to measure pancreatic injury markers (alpha-amylase and lipase activity) and lipid markers (total cholesterol (TC), high-density lipoprotein lipase (HDL-cholesterol), low-density lipoprotein lipase (LDL-cholesterol) and triglyceride levels (TG)). Then animals were sacrificed and pancreatic tissues were collected and froze at -80 °C for estimation of tissue oxidative stress markers including; reduced glutathione (GSH), and malondialdehyde (MDA) levels. Moreover, pancreatic TNF-alpha and myeloperoxidase levels were also measured to estimate the immune cells infiltration in the pancreas. All biochemical tests were conducted in duplicate. For histological evaluation, pancreatic tissue samples were stored in 10% formalin.

Assessment of pancreatic injury markers:
According to the reported method [32] serum amylase and lipase activities were measured using commercial kits obtained from Spectrum Diagnostic (Egypt) and Quimica Clinica Aplicada S.A (Greece), respectively.

Assessment of Lipid markers:
Serum total cholesterol, HDL-cholesterol, LDL-cholesterol and triglyceride levels were measured according to Allain et al. [33] using kits obtained from Spectrum Diagnostic, Egypt.

Assessment of pancreatic immune cells infiltration markers:
TNF-alpha and MPO levels were measured using ELISA kits according to the manufacturer's guidelines Ray Biotech, Norcross, GA, USA and MyBioSource, San Diego, CA, USA, respectively. These kits were selected due to their high value of sensitivity, specificity, and inter-assay and intra-assay accuracy.

Assessment of pancreatic oxidative stress markers:
Pancreatic GSH and MDA levels were measured by using the procedure of Ellman et al. [34] and Uchiyama et al. [35], respectively.

Histological Examination of Pancreatic tissue injury:
The pancreatic tissue from each animal was fixed in 10% formalin, dehydrated in alcohol, and fixed in paraffin wax. 5-μm thick sections stained with hematoxylin and eosin for the general morphology. Histological examinations were made by two pathologists who were blind to the experimental treatment.

Statistical Analysis:
Statistical analysis was carried out using IBM-SPSS statistics (version 20). The data were expressed as mean ± SE. Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey-Kramer (TK) multiple comparisons post-test, values of p < 0.05 were considered as significant.

Results:
A: Effect on pancreatic injury markers:
The group administered L-asparaginase showed a significant increase in pancreatic injury markers as serum alpha-amylase and lipase levels compared to the control group. Treatment with enoxaparin (100 IU/kg) or diclofenac (2 mg/kg) significantly decreased only the serum alpha-amylase level by 98.37% and 98.45% respectively (Table 1).

B: Effect on lipid markers:
Table 2 showed that L-asparaginase didn’t significantly alter serum TC, HDL-cholesterol or LDL-cholesterol. However, it significantly increased TG levels by 113.8%. It is to be noted that enoxaparin (100 IU/kg) when combined with L-asparaginase, only TG level significantly reduced by 80.6 %.

C: Effect on pancreatic immune cells infiltration markers:
L-asparaginase induced asignificant elevation in the pancreatic inflammatory markers; MPO and TNF-alpha levels compared to the control group. While rats treated with enoxaparin (100 IU/kg) or diclofenac (2 mg/kg) were protected against L-asparaginase induced rise in both MPO by 67.9 % and 63.2% respectively, and TNF-α by 62.1 % and 62 % respectively (Table 3)
**D: Effect on pancreatic oxidative stress markers:**

Rats took L-asparaginase revealed a marked depletion of pancreatic antioxidant defense system proved by aspecific decrease in reduced glutathione (GSH) when compared to the control group. Furthermore, L-asparaginase significantly elevated pancreatic lipid peroxides when compared to the control group. Rats treated with enoxaparin (100 IU/kg) or diclofenac (2 mg/kg) had significantly elevated GSH level by 119.3% and 143.8% respectively and significantly reduced MDA level by 87.2% and 85.4% respectively compared to the L-asparaginase group (Table 3).

### Table 1: Effect of enoxaparin and diclofenac on pancreatic injury markers

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Serum alpha - amylase activity (U/L) (mean ± SE)</th>
<th>Serum lipase activity (U/L) (mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline (Normal control)</td>
<td>1013.35 ± 1.77</td>
<td>33.59 ± 0.31</td>
</tr>
<tr>
<td>Enoxaparin</td>
<td>1021.46 ± 0.39</td>
<td>38.05 ± 0.56 ^</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>1020.96 ± 2.78</td>
<td>37.61 ± 1.44 ^</td>
</tr>
<tr>
<td>Enoxaparin + Diclofenac</td>
<td>1020.75 ± 2.75</td>
<td>37.91 ± 1.35 ^</td>
</tr>
<tr>
<td>L-asparaginase (Positive control)</td>
<td>1030.76 ± 3.33</td>
<td>41.8 ± 0.42 ^</td>
</tr>
<tr>
<td>L-asparaginase + Enoxaparin</td>
<td>1014.06 ± 0.71 ^</td>
<td>37.94 ± 0.47 ^</td>
</tr>
<tr>
<td>L-asparaginase + Diclofenac</td>
<td>1014.86 ± 1.16 ^</td>
<td>37.91 ± 0.73 ^</td>
</tr>
<tr>
<td>L-asparaginase + Enoxaparin + Diclofenac</td>
<td>1013.49 ± 1.18 ^</td>
<td>38.09 ± 0.99 ^</td>
</tr>
</tbody>
</table>

Each value represents the mean of 8 experiments ± SEM.

Multiple comparisons were done using one-way ANOVA followed by Tukey-Kramer as post-ANOVA test.

a: p< 0.05 compared with the normal control group  b: p< 0.05 compared with the positive control group

### Table 2: Effect on Lipid markers

<table>
<thead>
<tr>
<th>Treatments</th>
<th>TC (mg/dl) (mean ± SE)</th>
<th>HDL-cholesterol (mg/dl) (mean ± SE)</th>
<th>LDL-cholesterol (mg/dl) (mean ± SE)</th>
<th>TG (mg/dl) (mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline (Normal control)</td>
<td>59.88 ± 1.82</td>
<td>11.57 ± 0.43</td>
<td>36.17 ± 1.72</td>
<td>54.73 ± 0.59</td>
</tr>
<tr>
<td>Enoxaparin</td>
<td>56.18 ± 1.24</td>
<td>11.11 ± 0.53</td>
<td>32.04 ± 1.71</td>
<td>44.96 ± 2.19 ^</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>55.44 ± 1.17</td>
<td>10.88 ± 0.44</td>
<td>32.11 ± 0.84</td>
<td>49.05 ± 1.96</td>
</tr>
<tr>
<td>Enoxaparin + Diclofenac</td>
<td>56.5 ± 0.99</td>
<td>11.14 ± 0.43</td>
<td>32.73 ± 1.06</td>
<td>43.63 ± 1.07 ^</td>
</tr>
<tr>
<td>L-asparaginase (Positive control)</td>
<td>63.46 ± 2.34</td>
<td>12.7 ± 0.23</td>
<td>38.13 ± 2.18</td>
<td>62.31 ± 0.58 ^</td>
</tr>
<tr>
<td>L-asparaginase + Enoxaparin</td>
<td>55.56 ± 1.57</td>
<td>11.06 ± 0.45</td>
<td>31.6 ± 1.56</td>
<td>50.25 ± 1.62 ^</td>
</tr>
<tr>
<td>L-asparaginase + Diclofenac</td>
<td>62.59 ± 2.61</td>
<td>11.12 ± 0.38</td>
<td>38.81 ± 2.01</td>
<td>67.92 ± 1.45</td>
</tr>
<tr>
<td>L-asparaginase + Enoxaparin + Diclofenac</td>
<td>62.65 ± 2.34</td>
<td>11.67 ± 0.67</td>
<td>36.37 ± 2.36</td>
<td>51.96 ± 1.17 ^</td>
</tr>
</tbody>
</table>

Each group consists of 8 animals. The data are expressed as mean ± SE.

Multiple comparisons were done using one-way ANOVA followed by Tukey-Kramer as post-ANOVA test.

a: p< 0.05 compared with the control group  b: p< 0.05 compared with the pancreatitis group

### Table 3: Effect on oxidative stress and immune cells infiltration markers

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Oxidative stress markers (mean ± SE)</th>
<th>Immune cells infiltration markers (mean ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GSH level (µmol/g tissue)</td>
<td>MDA level (µmol/g tissue)</td>
</tr>
<tr>
<td>Saline (Normal control)</td>
<td>7.46 ± 0.36</td>
<td>58.79 ± 1.76</td>
</tr>
<tr>
<td>Enoxaparin</td>
<td>7.54 ± 0.25</td>
<td>58.05 ± 3.13</td>
</tr>
<tr>
<td>Diclofenac</td>
<td>7.64 ± 0.17</td>
<td>56.78 ± 0.68</td>
</tr>
<tr>
<td>Enoxaparin + Diclofenac</td>
<td>7.7 ± 0.2</td>
<td>58.49 ± 0.39</td>
</tr>
<tr>
<td>L-asparaginase (Positive control)</td>
<td>5.27 ± 0.25 ^</td>
<td>66.15 ± 1.14 ^</td>
</tr>
<tr>
<td>L-asparaginase + Enoxaparin</td>
<td>6.85 ± 0.33 ^</td>
<td>57.66 ± 0.46 ^</td>
</tr>
<tr>
<td>L-asparaginase + Diclofenac</td>
<td>7.58 ± 0.28 ^</td>
<td>56.51 ± 0.92 ^</td>
</tr>
<tr>
<td>L-asparaginase + Enoxaparin + Diclofenac</td>
<td>6.74 ± 0.34 ^</td>
<td>55.56 ± 1.26 ^</td>
</tr>
</tbody>
</table>

Each group consists of 8 animals. The data are expressed as mean ± SE.

Multiple comparisons were done using one-way ANOVA followed by Tukey-Kramer as post-ANOVA test.

a: p< 0.05 compared with the control group  b: p< 0.05 compared with the pancreatitis group

**Histological examination:**

Histological examination of pancreatic injuries due to L-asparaginases showed many changes in the pancreatic acini with no changes in the Langerhans cells. These changes appeared as microscopic foci of multivesicular cytoplasmic vacuoles, commonly noted in the paranuclear region. Nuclei were vesicular with distinct and prominent nucleoli. Mild inflammatory exudate was detected in some areas. The pancreatic parenchyma showed vacuolation of epithelial lining, with immune cells infiltration, lipid necrosis, and focal intraluminal hemorrhage (Figure 2). Treatment with enoxaparin and/or diclofenac showed protective effect of the pancreatic tissue as they ameliorated these changes (Figure 1).
**Fig. 1:** Effect of 5 days daily administration of diclofenac (2 mg/Kg) and enoxaparin (100 IU/Kg) alone or in combination on histopathological examination of pancreatic tissue in normal rats.

A, B and C: Enoxaparin sodium treated rats (100 IU/Kg, s.c., daily for 5 days), diclofenac sodium treated rats (2 mg/Kg, i.p., daily for 5 days) and enoxaparin sodium treated rats with diclofenac sodium treated rats respectively, showed normal pancreatic parenchyma with very mild vacuolation of epithelial lining pancreatic acini.

D: L-asparaginase treated rats (1000 IU/Kg, i.m., daily for 5 days) showed severe vacuolation of epithelial lining pancreatic acini.

E and F: L-asparaginase treated rats with either enoxaparin sodium treated rats or with diclofenac sodium treated rats respectively, showed moderate vacuolation of epithelial lining pancreatic acini.

G: L-asparaginase treated rats with enoxaparin sodium treated rats and diclofenac sodium treated rats, showed mild vacuolation of epithelial lining pancreatic acini.

H: L-asparaginase treated rats, showed severe inflammatory cells infiltration.

I: L-asparaginase treated rats with diclofenac sodium treated rats, showed mild inflammatory cells infiltration.
Fig. 2: Effect of 5 days daily administration of diclofenac (2 mg/Kg) and enoxaparin (100 IU/Kg) alone or in combination on histopathological examination of L-asparaginase induced pancreatitis in rats

J and K: L-asparaginase treated rats (1000 IU/Kg, i.m., daily for 5 days), showed severe necrosis of pancreatic acini, x 200 and x 400 respectively

L: L-asparaginase treated rats with diclofenac sodium treated rats (2 mg/Kg, i.p., daily for 5 days), showed mild necrosis of pancreatic acini

M: L-asparaginase treated rats showed severe focal pancreatic hemorrhage

N and O: L-asparaginase treated rats with either enoxaparin sodium treated rats (100 IU/Kg, S.C., daily for 5 days) or with diclofenac sodium treated rats respectively, mild focal pancreatic hemorrhage

P: All groups, showed intact islets of Langerhans cells

Q and R: Normal saline treated rats, showed normal pancreatic parenchyma architecture. Pancreatic parenchyma was closely packed acini

Discussion:

Acute pancreatitis in the cancer patient is likely to be caused by the same factors that are implicated in the general population, such as gallstones or alcohol abuse, etc.[36]. However, acute pancreatitis can also be a complication of either medical or surgical therapy as endoscopic retrograde cholangiopancreatography and splenectomy[36]. It is a recognized complication of a number of antineoplastic chemotherapeutic agents, although the drug with which it is most commonly reported is L-asparaginase. Other antineoplastic drugs that are known to induce pancreatitis are corticosteroids, 6-mercaptopurine, cytarabine, cisplatin, vincristine,
methotrexate, mitomycin C, cyclophosphamide, doxorubicin, and ifosfamide[37]. It is also a complication of other supportive medications such as anti-microbial agents (erythromycin, clarithromycin, and isoniazid), diuretics (furosemide, chlorothiazide, and hydrochlorothiazide), H2 blockers (cimetidine, ranitidine), proton pump inhibitors (omeprazole), cardiac agents (captopril, enalapril, losartan)[38]. Diagnosis of acute pancreatitis depends on both imaging studies by abdominal CT or US and blood markers as amylase and lipase activities[39]. Other non-specific markers like inflammatory mediators, cytokines, ROS, ammonia level, complete blood count, electrolyte imbalance, tissue myeloperoxidase have been used[40, 41]. Histopathological studies are the key marker for diagnosis and grading of the disease. It shows the change of the parenchymal cells morphology by auto-digestion due to inappropriately activated enzymes. Increases hemorrhage from the small vessel and subsequently thrombosis. Increases intraductal pressure, causing enzyme-rich interstitial fluid to accumulate, which causes fat necrosis and attracts immune cells that release cytokines, attacking acini and cause interstitial edema, which impairs blood flow and causes ischemia and acinar cell injury[42].

Clinical data suggested that enoxaparin, a low molecular weight heparin, appears to be a potentially viable option for treating acute pancreatitis [43]. Enoxaparin is an anticoagulant drug used for the treatment of thrombosis and other hemodynamic instabilities. Enoxaparin possesses anti-inflammatory, lipid lowering, and anti-oxidant properties[14, 15]. In vivo studies indicated that enoxaparin is a potentially protective against different models of acute pancreatitis by several mechanisms as inhibition of the release of nuclear factor NF-κB, inhibiting release of pro-inflammatory cytokines like (TNF)-alpha, IL-8, IL-6 and IL-1b, and reducing the release of tissue MPO[44].

Diclofenac, a NSAID, appears to be a probable convenient choice for treating acute pancreatitis [45]. Diclofenac, a phenylacetic acid derivative, has anti-inflammatory, anti-oxidant, anti-angiogenic, voltage-dependent calcium channels blocker properties [27, 46], but these properties depend on the amount of the drug used in doses less than 50mg/kg considered to be protective with anti-oxidant activity, doses more than 150 mg/kg considered toxic doses and doses more than 400 mg/kg considered life-threatening dose[27, 47, 48]. In vivo studies indicated that diclofenac is a possibly protective against different models of acute pancreatitis by inhibitory effect on phospholipaseA2 (PLA2), TNF-alpha, and L-selectin, also it increases the effect of Lipoxin A4 (LXA4), Resolvin D1 (Rvd1), and Resolvin E1 (RvE1) levels, which responsible for reducing inflammation[49, 50].

So, the hypothesis of this study is to compare two drugs, which are used during supportive treatment of leukemia, in the prevention of initiation and dissemination of pancreatitis during treatment with L-asparaginase. The hypothesis depends on the anti-inflammatory activity of the drugs. Also, reflects and evaluates of other properties such as antioxidant and lipid-lowering properties on the protection of pancreatic parenchyma during treatment with L-asparaginase.

In the present study, administration of either enoxaparin or diclofenac didn’t change the serum alpha-amylase activity in normal rats, but significantly increase the serum lipase activity (Table 1). By administration of L-asparaginase serum alpha-amylase and lipase activity significantly increased, this is due to the effect of direct destruction of the pancreatic parenchyma, by inflammation and tissue auto-digestion, and increasing the pancreatic intraductal pressure causing enzyme-rich interstitial fluid to accumulate. These results are interconnected with previous studies [51]. Co-treatment of animals with either enoxaparin or diclofenac, the activity of serum alpha-amylase only returned to the normal levels. The possible mechanism of action for this effect is the ability of enoxaparin to restore the properties of pancreatic microcirculation and blood rheology that involved in the development of the inflammatory response during acute pancreatitis, therefore, positive effects on tissue perfusion[11, 12]. Diclofenac reduced the release of PLA2, which plays a critical role in the initiation and propagation of the inflammatory response during acute pancreatitis, as during the inflammation activated PLA2 causes coagulation and necrosis of pancreatic tissue [52]. The current results are in good agreement with earlier studies which confirmed the protective role of both enoxaparin and diclofenac against different models of acute pancreatitis in rats [52].

L-asparaginase didn’t change the level of TC, HDL, and LDL but increased the triglycerides level (Table 2). These findings were different from other clinical studies in which TG, TC and LDL levels increased and HDL level decreased during L-asparaginase treatment, but these studies used corticosteroids and other chemotherapeutic agents during treatment which may cause an imbalance in lipid metabolism and increase their levels[53]. Enoxaparin effect arises from its ability to release lipoprotein lipase from vascular endothelium, therefore reduction of TG [54]. Also, it reduces lipoprotein lipase degradation, with increases its quantitative secretion from adipocytes [55], and increase in the expression of hepatic triglyceride lipase, which is involved in hepatic clearance of chylomicron and VLDL found in the blood[56].

In an attempt to investigate the possible protective effect of enoxaparin or diclofenac, different markers of oxidative stress were examined. Oxidative stress produced by free radicals inside the pancreatic cells is being believed to have a direct destructive effect of the tissue[57, 58]. In our study reduced glutathione and malonaldehyde were measured to evaluate the production of oxidative stress during L-asparaginase treatment and to evaluate the antioxidant activity of both drugs Table 3. The results showed that either enoxaparin or
diclofenac didn’t induce oxidative stress in the pancreatic tissue in normal rats. The enzyme significantly induced oxidative stress and lipid peroxidation reflected by reduced the GSH and increased MDA levels as showed in many experimental studies[59]. These effects were mainly due to the increase in the production of reactive oxygen species through local immune cell infiltration, which induces toxic oxidized mediators and therefore disseminated inflammation[60]. By simultaneous applying of either enoxaparin or diclofenac, the oxidative parameters returned to the normal levels. Enoxaparin has an ability to regulate endothelial NO level, stabilizing vascular endothelium, enhance the antioxidant activity of superoxide dismutase, reduce the activation of NF-κB, and inhibiting intracellular Ca2+ release by inhibiting Ca2+-ATPase pump[61, 62]. In addition, diclofenac significantly reduced oxidative stress and lipid peroxidation. This antioxidant effect rises from several pathways, its ability to scavenge the free radicals 1,1-diphenyl-2-picrylhydrazyl (DPPH), decrease the production of on superoxide anion (O2−), peroxynitrite, oxy and lipid-radical, hydroxyl radicals (•OH) and Fe2+/ascorbate system with some effect on NADPH-dependent LPO [25]. It also inhibits the production of hypochlorous acid[46]. These results are in accordance with prior studies of[25, 63].

Assessment of immune cells infiltration of pancreatic tissue revealed that either enoxaparin or diclofenac didn't significantly elevate the level of pancreatic MPO or TNF-alpha in normal animals, but these levels increased significantly during L-asparaginase administration(Table 3). These results are in accordance with previous studies which confirmed the role of immune cells infiltration of pancreatic tissue in the progression of acute pancreatitis[29]. By concurrent administration of enoxaparin or diclofenac, the parameters returned to the normal levels. A possible mechanism of action for this effect is the inhibitory effect of enoxaparin on the binding of MPO to endothelial cells, reduction of endocytosis of MPO into the tissues, and increase the plasma level of immobilized MPO due to forcefully liberated the enzyme from its vascular stores [61, 64, 65]. This data comes in similarly with the studies performed by Joanna et al. [66] and Baldus et al.[67]. The reduction of the TNF-alpha could be attributed to inhibition of TNF-alpha activity and increased its receptor solubility[68]. Diclofenac significantly reduced the tissue MPO level. This is may be due to inhibition of leucocyte chemotaxis, and reduction in the intracellular content of ATP by shedding of L-selectin, which responsible for cellular adhesion and MPO activation[69]. Diclofenac also reduced TNF-alpha level by reduction of cyclooxygenases and lipid peroxidation, which are responsible for induction of cytokines, and inhibition of serine metalloproteinase which is responsible for cytokine secretion[70].

Histopathological examination, the gold criterion for the diagnosis of acute pancreatitis, revealed that L-asparaginase resulted in many changes in the pancreatic acini with no changes in the Langerhans cells. These changes appeared as microscopic foci of multivesicular cytoplasmic vacuoles, commonly noted in the paranuclear region. Nuclei were vesicular with distinct and prominent nucleoli. Mild inflammatory exudate was detected in some areas. The pancreatic parenchyma showed vacuolation of epithelial lining, with immune cells infiltration, lipid necrosis, and focal intraluminal hemorrhage. Treatment with enoxaparin and/or diclofenac ameliorated most of these changes and showed a protective effect of the pancreatic tissue. The current results are in parallel with previous studies which confirmed the protective effects of both enoxaparin and diclofenac against different models of acute pancreatitis in rats[29, 51]. Pancreatic sections from control group stained with H & E showed normal pancreatic parenchyma architecture. Pancreatic parenchyma was closely packed acini. The secretions of the acini were empty into ducts, lined with a simple low cuboidal epithelium, which becomes stratified cuboidal in the larger ducts. Treatment with either enoxaparin sodium alone, diclofenac sodium alone, or two drugs combined showed only mild vacuolation of epithelial lining pancreatic acini (Figure 1). On the contrary, pancreatic parenchyma sections from L-asparaginase treated group (Figure 2) showed moderate to severe inflammatory cells infiltration, severe vacuolation of epithelial lining pancreatic acini, severe necrosis of pancreatic acini and severe focal pancreatic hemorrhage. Sections from the group treated with L-asparaginase and enoxaparin sodium concomitantly displayed moderate vacuolation of epithelial lining pancreatic acini with mild focal pancreatic hemorrhage and without inflammatory cells infiltration or necrosis of pancreatic acini. Sections from the group treated with L-asparaginase and diclofenac sodium concomitantly displayed moderate vacuolation of epithelial lining pancreatic acini with mild inflammatory cells infiltration, mild necrosis of pancreatic acini and mild focal pancreatic hemorrhage. Sections from the group treated with L-asparaginase, diclofenac sodium, and enoxaparin sodium showed only mild vacuolation of epithelial lining pancreatic acini. All drugs do not change the histological characteristics of endocrine part of thepancreas that contains islets of Langerhans.

It is now known that the administration of both enoxaparin and diclofenac together with L-asparaginase didn't significantly change the activities of serum alpha-amylase and lipase, levels of GSH and MDA, levels of MPO and TNF-alpha as compared from each drug alone. But, during treatment of L- asparaginase rats with enoxaparin in combination with diclofenac, serum triglyceride level decreased and it was found that this combination gave the superior effect on serum triglyceride level than diclofenac alone.

Collectively, the present study provides evidence for pancreatic tissue protection of enoxaparin and diclofenac. The mechanism of underlying these promising effects involve attenuating pancreatic tissue injuries, reduce oxidative stress, decrease immune cell infiltration and restore lipid parameters to normal levels.


