ORIGINAL ARTICLE

Preventive effects of enoxaparin and hesperidin in cerulein-induced acute pancreatitis in rats

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Background/aims: *Acute pancreatitis accounts for almost 250.000 hospital admissions annually in the United States. Most promising treatment approaches are preventive; however, little is known about the early factors initiating acute pancreatitis. We aimed to evaluate the preventive effects of enoxaparin and hesperidin in cerulein-induced acute pancreatitis.* **Patients and methods:** *We used 70 Wistar albino rats for this study. Rats were divided into 7 groups: control group, and groups that were administered cerulein (Group 2), enoxaparin (Group 3), hesperidin (Group 4), cerulein with enoxaparin (Group 5), cerulein with hesperidin (Group 6), and cerulein with both enoxaparin and hesperidin (Group 7). Edema formation; leukocyte infiltration; measurement of the amylase level, pancreatic tissue weight, and pancreatic tissue oxidative capacity; and chemiluminescence using luminol, lucigenin, and nitric oxide levels as indices of tissue oxidative capacity were used to evaluate pancreatitis.* **Results:** *Acute edematous mild pancreatitis was induced in groups 2, 5, and 6 by cerulein injections. Enoxaparin and hesperidin significantly decreased (p < 0.001) all the tested parameters in these rats. Enoxaparin and hesperidin did not offer complete protection but showed 50% decrease in edema formation. The preventive agents showed no superiority to each other. Further, when enoxaparin and hesperidin were used in combination, no significant additive effects with regard to anti-inflammatory and anti-oxidative actions were present.* **Conclusion:** *We showed that both enoxaparin and hesperidin exerted significant preventive effects in all the parameters related to acute pancreatitis in our experimental rat model.*

Key words: Acute pancreatitis, enoxaparin, hesperidin

Serulein ile indüklenen akut pankreatit modelinde ratlarda enoksaparin ve hesperidinin prevantif etkileri

Giriş ve Amaç: *Amerika Birleşik Devletleri'nde yıllık 250.000 hastane başvurusu akut pankreatit nedeniyle olmaktadır. En çok umut bağlanan tedavi şekilleri prevantif yaklaşımlar olmasına rağmen, pankreatiti başlatan ilk faktörler yeteri kadar bilinmemektedir. Biz bu çalışmada, Serulein ile indüklenen akut pankreatit modelinde enoksaparin ve hesperidinin preventif etkilerini değerlendirmeyi amaçladık.* **Materyal ve Metod:** *Çalışmada 70 Wistar albino rat kullandık. Ratlar 7 gruba bölündü: kontrol grubu, ve; serulein (Grup 2), enoksaparin (Grup 3), hesperidin (Grup 4), serulein ve enoksaparin (grup 5), serulein ve hesperidin (grup 6), serulein, enoksaparin ve hesperidin (grup 7) verilen gruplar. Pankreatiti değerlendirmek üzere; ödem oluşumu ve lökosit infiltrasyonu; amilaz seviyesi, pankreatik doku ağırlığı ve pankreatik doku oksidatif kapasitesi ölçümleri kullanıldı. Oksidatif kapasite ölçümü için kemiluminesans tekniği kullanılarak dokuda luminol, lusigenin, ve nitrik oksit seviyeleri ölçüldü.* **Bulgular:** *Grup 2, 5, 6 ve 7'de, serulein injeksiyonları ile akut ödematöz hafif pankreatit oluştu. Enoksaparin ve hesperidin kullanıldıkları ratlarda, tüm çalışma parametrelerini anlamlı derecede (P < 0.001) düşürdü. Enoksaparin ve hesperidin tam bir prevansiyon sağlamamakla beraber, ödem oluşumunda %50 azalma sağladılar. Çalışmada kullanılan prevantif maddeler birbirlerine bir üstünlük göstermediler. Ayrıca, enoksaparin ve hesperidin, birlikte kullanıldığında, anti-inflamatuvar ve anti-oksidatif etki anlamında katkı arttırıcı anlamlı bir etki göstermediler.* **Sonuç:** *Bu eksperimental çalışmada; enoksaparin ve hesperidinin, rat modelinde, akut pankreatitle ilgili kullanılan tüm parametrelerde, anlamlı prevantif etkilerini gösterdik.*

Anahtar kelimeler: Akut pankreatit, enoksaparin, hesperidin

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INTRODUCTION

Acute pancreatitis is a common disorder that has an annual incidence of 5-80 per 100.000 individuals (1). The pathogenesis involves a complex interplay of environmental and, as yet incompletely characterized, genetic factors. The causes of acute pancreatitis have been well-documented, and when no immediate cause is found, it is generally ascribed to alcohol misuse, gallstones, miscellaneous factors, and idiopathic causes. Endoscopic retrograde pancreatography, hypertriglyceridemia, hypercalcemia, and autoimmune pancreatitis are the other well-recognized etiological factors in acute pancreatitis (1,2). Acute pancreatitis is a gradual inflammatory process that affects the pancreas as well as the surrounding tissues or remote organ systems and has high mortality rate ranging from 15 to 25% (3). The severe form of the disease is characterized by a significant systemic inflammatory response, which may be associated with organ dysfunction. Its pathogenesis is poorly understood, and multiple factors and pathways have been suggested to initiate and perpetuate this complex disease. Acute pancreatitis is characterized by inflammation, oxidative stress, interstitial edema, impaired perfusion, ischemia, and parenchymal necrosis (3-5). Inflammatory cells such as polymorphonuclear cells, monocytes, macrophages, and lymphocytes; endothelial cells; and thrombocytes play important roles in the inflammatory aspects of pancreatitis, whereas the impaired perfusion and oxidative stress caused by excess reactive oxygen species (ROS) result in tissue damage (4-8). Ischemia alone and decreased capillary perfusion are well-known factors that either directly cause severe pancreatitis or enable the progression of pancreatitis from mild to severe forms (3). Prevention and treatment primarily involve tissue protection by improving capillary blood flow, preventing thrombosis, and reducing inflammation (3,4,9). In this study, we used an animal model to test the therapeutic potential of early drug treatment in reducing the progression of acute pancreatitis.

The currently known topical pathways are insufficient to fully explain the complex process of acute pancreatitis. Moreover, current treatments cannot adequately cure the condition in patients. Therefore, further research is required to investigate the pathogenesis and to improve the treatment modalities in acute pancreatitis.

Cerulein (CRL) is a cholecystokinin analogue that affects acinar cells, leading to generation of excessive ROS and is used to induce pancreatitis in experimental models. CRL-induced pancreatitis is highly preferred in experimental models of pancreatitis. The ROS extensively affect pancreatic tissue, resulting in oxidative stress by lipid peroxidation and denaturation of enzymes and proteins, which consequently leads to disturbance in the cell membranes and proteolytic digestion of the cell components in acute pancreatitis (4,8,10,11).

Here, we investigated the effects of enoxaparin (ENX) and hesperidin (HES) in a model of experimental CRL-induced pancreatitis. ENX is a low-molecular-weight heparin that is widely used in surgery. ENX is a potent vasodilator, and it inhibits platelet aggregation and thrombosis, increases capillary permeability and perfusion effectively, and scavenges ROS considerably (12-16). There is no consensus in literature concerning the effects of anticoagulants in inflammation and pancreatitis. HES is a flavonoid with strong anti-inflammatory effects. It is a powerful ROS scavenger that increases tissue perfusion and functions as a microcapillary regulator (17–22). However, no studies have yet investigated the use of HES for treating patients with acute pancreatitis.

In this study, the preventive effects of ENX and HES due to their powerful anti-oxidative, anti-inflammatory, and positive effects on tissue perfusion potentials have been investigated on experimental acute pancreatitis model. Furthermore, the probable additive effects of ENX and HES because of their similar mechanism of actions have been searched thoroughly.

MATERIALS and METHODS

In this study, we used 70 male Wistar albino rats weighing 200-250 g. The rats were fed a standard diet (24% protein, 1% lysine, 0.6% methionine, 0.4% cysteine, 7% cellulose,

1% NaCl, 1-2% Ca, and 2.650 kcal/kg energy) and water ad libitum throughout the experimental period.

All the animals were housed in standard polypropylene cages maintained under standard conditions of 22–24°C temperature and a light/dark cycle of 12 h each. The rats were divided into 6 groups of 10 animals each. Group 1 was designated as the Sham control group; the rats of this group did not undergo any treatment/procedure and were injected with saline injections alone. The

rats from Group 2 were administered CRL alone. Groups 3 and 4 received ENX and HES, respectively. CRL-induced rats in Groups 5 and 6 received ENX (CRL + ENX) and HES (CRL + HES), respectively. Group 7 rats were injected with ENX, HES, and CRL (CRL + HES + ENX).

Chemicals and Methods of Application

Cerulein: CRL was obtained from Sigma (St. Louis, MO, USA). CRL was dissolved in distilled water (16.3 μg/mL) and applied to the dorsal skin of the rats subcutaneously as described in the literature (23). Pancreatitis was induced by injecting 50 μg/kg CRL at hourly intervals for a total of 7 times.

Enoxaparin: ENX (Clexane) was obtained from Eczacıbası Rhone-Poulenc (Istanbul, Turkey). ENX was applied to the dorsal skin of the rats subcutaneously at the standard, commonly used dose of 100 U (1 mg/kg) (12,13). This clinical anticoagulation dose of ENX was also used in an experimental pancreatitis model of rats in a previous study (14). ENX was injected into each animal only once, just before the first CRL injection.

Hesperidin: HES was obtained from Sigma (St. Louis, MO, USA). Hesperidin was dissolved in distilled water (1% w/v), and 0.3 g/kg was applied subcutaneously to the dorsal skin of the rats. In previous studies on an anti-inflammatory skin model of mice, this dose was determined to be an effective anti-inflammatory dose of HES (21,22). HES was injected in each animal only once, just before the first CRL injection. The HES solution was maintained at 4°C and protected from light.

Animal Protocol and Sample Collection

A detailed description of this study's methodology was published in our previous study (24). CRL and 0.6 mL saline were injected at hourly intervals into the rats in Group 2 and in the Sham Group, respectively, for a total of 7 times per group. At the beginning of the study, Groups 3 and 4 received injections of ENX and HES, respectively. Groups 5 and 6 received ENX and HES injections, respectively, just before the CRL injections, which were administered at hourly intervals for a total of 7 times. Finally, Group 7 received injections of ENX and HES together just before the CRL injections, which were administered at hourly intervals for a total of 7 times. All the 70 animals were fasted for 12 h prior to the onset of the experiments and during the additional 7 h during which they received the hourly injections. Then, food was provided ad libitum for 2 h thereafter, following which they

were sacrificed for blood and tissue collection. This protocol ensures that all the animals had access to the same amount of food during the experiment, thereby alleviating the effects of nutrient absorption on the development of acute responses. The animals were sacrificed by decapitation 9 h after the first injections were administered. Blood samples for amylase analyses were collected from mixed arteriovenous blood after the animals were sacrificed. The blood samples were centrifuged at 10.000 g for 10 min. The serum amylase concentration was determined using the Phadebas amylase test (Pharmacia, Piscataway, NJ, USA). The pancreata were completely removed after decapitation. Two pieces (1 cm²) of pancreatic tissue were excised from the removed pancreas. One of the 1-cm² tissue sections was fixed overnight at room temperature in 10% formalin buffered to pH 7.0 with 0.1 M phosphate buffer for histopathological evaluations. Tissues were subsequently embedded in paraffin sections, cut into 5-mm slices, and stained with hematoxylin and eosin. The sections were analyzed and graded by a pathologist, who was blinded to the experimental design. The second 1-cm² pancreatic tissue section was weighed, dried at 60°C for 72 h, and weighed again. The differences between the wet and dry weights were recorded as the pancreatic fluid. The remaining pancreas tissues were preserved in ice bags to directly measure the free radical activity of the pancreas using chemiluminescence.

Assessment of Edema

The level of edema in the pancreatic tissue was graded as mentioned in literature-Grade 0: no tissue edema, grade 1: swelling of the cells, grade 2: interlobular edema, grade 3: moderate interlobular and intraacinar edema, and grade 4: severe interlobular and intraacinar edema (25).

Assessment of Leukocyte Infiltration

The neutrophilic inflammatory response was graded from 0 to 3 on the basis of the number and distribution of neutrophils infiltrating the pancreatic tissue samples (25). The grades were defined as follows: Grade 0, few granulocytes in the intravascular lumen; grade 1, intravascular margination of granulocytes in the capillary and postcapillary venules; grade 2, granulocytes present in the perivascular tissue; and grade 3, diffuse infiltration of the entire pancreatic gland.

Chemiluminescence

Measurements were made using a liquid scintilla-

tion counter (Tricab 1500; Packard Instruments, Downers Grove, IL, USA) in out-of-coincidence mode with a single active photomultiplier tube. Fresh pancreatic samples were gently transferred to vials, and luminescence was recorded at room temperature after adding 0.2 mM lucigenin or 0.2 mM luminal to the samples. The 2 chemiluminescence probes differed in selectivity. Luminol detects mainly H_2O_2, OH, hypochlorite, peroxynitrite, and lipid peroxy radicals, whereas lucigenin is particularly selective to the superoxide radical. Counts were obtained at 1-min intervals, and the results were obtained as the area under the curve for a counting period of 60 min, corrected for tissue weight (cpm/mg tissue). Detection of NO was based on the chemiluminescence reaction between NO and the purified luminal-H_2O_2 system (26).

Statistical Analysis

All the data were expressed as the mean±SD/SEM and 95% CI. The significance of the differences between the experimental groups for all parameters was estimated by one-way ANOVA, Tukey's test, and Kruskal-Wallis test where appropriate. The differences were considered statistically significant at $p < 0.05$. SPSS 16.0 for Windows (SPSS Inc, Chicago, IL, USA) was used for the analyses.

RESULTS

None of the rats died during the experiment. CRL injections led to threefold increase in serum amylase levels in group 2 in comparison with the control group ($p < 0.001$). ENX and HES, when used alone, did not affect the mean amylase levels. The ENX, ENX + HES, and HES groups showed significant inhibition ($p < 0.001$) in the CRL-induced amylase levels. However, while the ENX and ENX + HES inhibited amylase completely, incomplete inhibition was achieved with HES. The combined use of ENX and HES in group 7 had no additive effect in decreasing the amylase levels (Figure 1A).

The pancreatic tissue weights increased twofold after the CRL injections in group 2 compared to the control group ($p < 0.001$). ENX and HES when used alone did not significantly affect the pancreas tissue weights. ENX, HES, and ENX + HES inhibited the CRL-induced pancreatic weight increase in Groups 5, 6, and 7 ($p < 0.001$). No additive effects were observed with respect to the pancreatic weights when the HES and ENX were used in combination in group 7 (Figure 1B).

The lucigenin, luminol, and nitric oxide measurements revealed that the tissue oxidative capacity was higher in the CRL groups than in the control group ($p < 0.001$). ENX and HES when used alone did not cause any significant difference in the tissue oxidative capacity as compared to that in the control group. CRL induced nitric oxide, and the lucigenin levels were completely inhibited by all the preventive agents in Groups 5, 6, and 7 ($p < 0.001$). However, while ENX and ENX + HES caused complete inhibition and decrease ($p < 0.001$) of the CRL-induced mean luminol levels in Groups 5 and 6, only incomplete inhibition of luminol was achieved with HES in Group 6. The combined used of preventive drugs exhibited no additive effects in the inhibition of tissue oxidative capacity (Figure 1C-E).

The leukocyte infiltration was 8 times higher in the CRL group than in the control group ($p < 0.001$). ENX and HES when used alone caused no significant difference in the leukocyte infiltration. When CRL, ENX, and HES were used in various combinations, the CRL-induced leukocyte infiltration decreased significantly in Groups 5, 6, and 7. However, the inhibitory effects of preventive agents were not complete. When CRL, ENX, and HES were used in combination in Group 7, no additive effect was seen in leukocyte invasion (Figure 1F).

In this animal model of acute pancreatitis, tissue edema showed a fivefold increase in the CRL group as compared to the control animals (Figure 1G). While ENX or HES alone did not exert any effects, they reduced the CRL-mediated edema by more than 50%. These data suggest that both these drugs preserve capillary integrity during the development of acute pancreatitis.

DISCUSSION

CRL is a decapeptide derived from the skin of the *Hyla caerulea* frog, and it bears a strong resemblance to the C-terminal octapeptide of cholecystokinin (27). Previous studies have reported that sequential injections of CRL induce mild and reversible pancreatitis limited to the pancreas (11,23). This non-fatal experimental pancreatitis model is widely used in research of cellular biological events and to investigate the pathogenesis of pancreatitis (10,11,23,24). Our study demonstrates that CRL initiates the development of all major parameters of acute pancreatitis in rats, including tissue edema, amylase overproduction, inflammation, and oxidative stress (1,2,10,11).
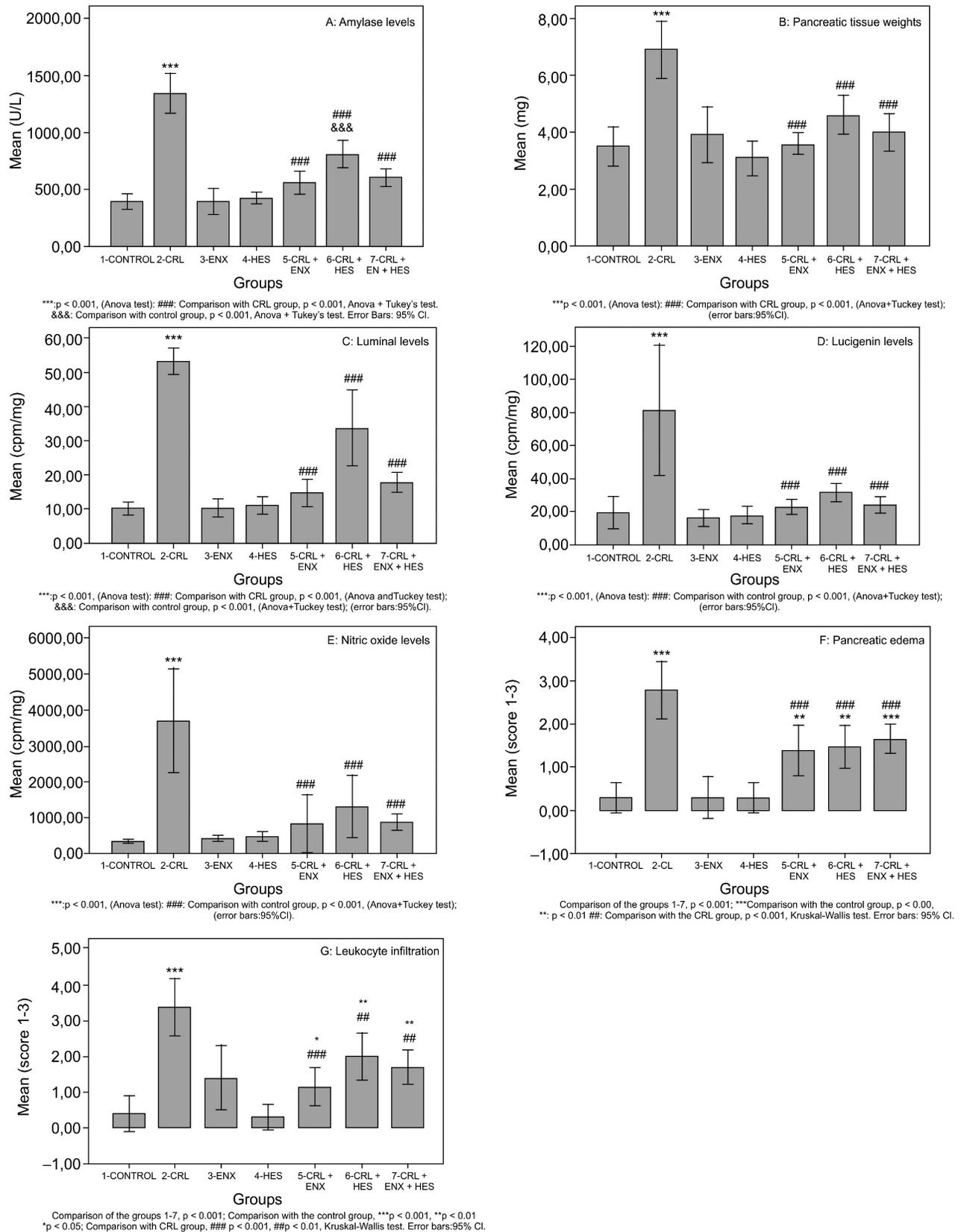
Figure 1. Comparison of amylase levels **(A)**; pancreatic tissue weights **(B)**; luminol **(C)**, lucigenin **(D)**, and nitric oxide levels **(E)**; and edema **(F)** and leukocyte infiltration scores **(G)** among the groups.

ROS are important mediators in many biological processes, including inflammation and tissue damage observed in pancreatitis. Strong evidence suggests that oxidative stress occurs during the course of acute pancreatitis (4,5). The superoxide-dependent chemotactic factors appear to play a critical role in the development of neutrophil-mediated inflammatory response. Nicotinamide adenine dinucleotide phosphate (NADPH)-related oxidase systems in the neutrophil membrane are activated by cytokines to transform oxygen to toxic metabolites such as superoxide, peroxide, and hydroxyradicals. Unstable and highly reactive toxic metabolites of molecular oxygen cause extensive damage to tissue, resulting in lipid peroxidation and enzyme and protein denaturation (4,8,24,28-32). Further, ROS act on the arachidonic acid system and increase thromboxane production, which impairs tissue perfusion. ROS also enhance the production of leukotriene B4, which activates leukocytes and mediates the discharge of lysosomal enzymes (8). In the present study, lucigenin, luminol, and nitric oxide were used for quantification of ROS using chemiluminescence. Luminol detects mainly hydrogen peroxide, hydoxyl, hypochlorite, peroxynitrite, and lipid peroxy radicals, whereas lucigenin is selective to the superoxide radical. The NO radical with an unpaired electron is one of the most potent toxic radicals in biological systems. It reacts rapidly with the superoxide radical, forming the highly reactive peroxynitrite anion (33). The ROS in the pancreatic tissues were significantly higher in CRL-induced rats than in the control animals in our study, and our results conform to those previously reported in literature (4,8,24).

This study demonstrates the powerful therapeutic potential of the concomitant use of 2 agents-HES (17-19) and ENX (12,13,15)-that are clinically applied for their anticoagulant or anti-inflammatory properties. To our knowledge, ours is the first study where HES was used to treat acute pancreatitis. Preventive use of both HES and ENX revealed significant reductions in CRL-induced alterations, including inflammation, amylase overproduction, and oxidative stress. Our study also revealed that both these drugs did not lead to any side effects in rats that were not administered CRL.

The effects of ENX and HES in pancreatitis are debated in literature. The mechanism of the preventive effects of these drugs is complex and includes several pathophysiological mechanisms that have not yet been fully clarified. ENX inhibits leukocyte adhesion by stabilizing the endothelial cells. Further, ENX reduces the secretion of endothelin-1, which plays an important role as a strong vasoconstrictor in the progression of pancreatitis from the mild to severe form. Focusing on acute pancreatitis, anticoagulants may exert their preventive effects by improving the microcirculation (14,34). In addition to the anti-oxidant effect and increased perfusion, anticoagulants may have direct anti-inflammatory effects (14,35,36) by inducing intracellular signaling through protease-activated receptors (37,38) or by inhibiting Mac-1 and ICAM-1 molecules (39-43).

On the other hand, as a member of the bioflavonoids, HES has antioxidant, anti-inflammatory, and anti-neoplastic effects (17,18). HES has been reported to be an effective anti-oxidizing agent that can scavenge ROS (44). It is efficacious both in the proliferative and exudative phases of inflammation. In addition to its microcapillary regulatory functions, it inhibits proinflammatory mediators, inducing chemotaxis of leukocytes (19,20,22). Previous studies have demonstrated a strong anti-inflammatory effect by pretreatment with hesperidin in mice ear edema. The inhibition of the inflammatory response was demonstrated by decreased leukocyte infiltration, epidermal thickness, and number of epidermal layers (21).

The effects of HES and ENX were equivalent in all the measured parameters of our study. Both these drugs reduced all the parameters of acute pancreatitis by nearly 50%, in agreement with their anti-inflammatory and/or antioxidant properties (12-14,17-22,35,36,44). The possible additive effects of ENX and HES were also investigated in this study. No significant additive effect of ENX + HES vs. HES or ENX on any of the parameters measured was observed. These results may suggest that HES and ENX have the same mechanism of action in the prevention of experimental acute pancreatitis. However, incomplete reductions in some of the CRL-induced inflammation and oxidative stress parameters of our experiment may suggest the inadequate effects ENX and HES or allude to other factors that play a role in acute pancreatitis that could not be effectively addressed by ENX and/or HES.

In conclusion, ENX and HES were evaluated as considerably potent preventive agents in this experimental pancreatitis model. However, further investigations are required to establish their roles in the pathogenesis of acute pancreatitis.

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