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The Effect of Erythropoietin in Acute Pancreatitis: An Experimental Study

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Background: Although the pathogenesis of acute pancreatitis is not clearly known, the most frequently mentioned factors are proinflammatory cytokines and nitric oxide (NO), which is stimulated by bacterial endotoxins. The relationship of erythropoietin (EPO), which is an erythroid line growth hormone, with proinflammatory cytokines and NO is also not clear. EPO has a well-known general tissue protective effect, yet its role in an inflammatory presentation like that of acute pancreatitis has not been investigated and documented. Therefore, the aim of this study was to investigate the role of EPO in acute pancreatitis.

Materials and Methods: 60 Wistar Albino rats were divided into 4 groups as follows: laparotomy (I), pancreatitis (II), pancreatitis + simultaneous application of EPO (III) and finally pancreatitis + administration of EPO after 3 hours (IV). The groups were further separated to subgroups by sacrificing the animals at 3, 6 and 24 hours. Pancreatitis was induced by injecting 4.5% sodium (Na)-taurocholate (0.1 ml / 100 g) into the pancreatic canal. Leukocyte counts and serum amylase and C-reactive protein (CRP) levels were measured. Histopathological changes of the pancreas were evaluated according to Spormann scores.

Results: EPO was shown to increase leukocyte counts in the early phase ($P < 0.05$) though the effect diminished in time. It also had an increasing effect on amylase in the early hours ($P < 0.005$), but the difference between the groups disappeared in the following hours. No effect was demonstrated on serum CRP. Pancreatic tissue revealed moderate degree of pancreatitis in group III sacrificed at 6 hours and in group IV evaluated at 6 and 24 hours. Despite the absence of a difference in the numerical analysis of Spormann scoring ($P > 0.05$), moderate pancreatitis developed in the group receiving EPO.

Conclusions: The results we have obtained demonstrate that more controlled models (employing low rates of sodium taurocholate or edematous pancreatitis) are necessary to better investigate the efficacy of EPO.

Key Words: Acute pancreatitis, experimental model, histopathology, erythropoietin

Eritropoetinin Akut Pankreatit Üzerine Etkisi: Bir Deneysel Çalışma

Amaç: Akut pankreatitin patogenezi net olarak ortaya konamamakla beraber en çok üzerinde durulan, proinflamatuar sitokinler ve bakteriyel endotoksinlerin stimule ettiği nitrik oksittir. Bir eritroid seri büyüme hormonu olan eritropoetinin (EPO) ise proinflamatuar sitokinlerle ve NO ile ilişkisi net değildir. EPO' nun genel doku protektif etkisi olduğu bilinmekle beraber akut pankreatit gibi inflamatuar bir tabloda nasıl etki gösterdiği araştırılmamıştır ve bilinmemektedir. Bu çalışmanın amacı akut pankreatitte EPO'nun etkisini araştırmaktır.

Yöntem ve Gereç: 250-300 gr ağırlığında 60 adet Wistar Albino rat 4 gruba ayrıldı. Bunlar, (I) Laparotomi, (II) pankreatit, (III) pankreatit + eşzamanlı EPO uygulanan ve (IV) pankreatit + 3 saat sonra EPO uygulanan gruplardı. Her grup ayrıca 3., 6., 24. saatlerde sakrifikasyon için ayrıldı. Pankreatik kanala % 4.5 Na taurokolat (0.1 ml / 100 gr) injeksiyonu ile akut pankreatit oluşturuldu. Lökosit, amilaz, CRP seviyesi ve pankreasta (Spormann skorlamasına göre) histopatolojik değişiklik araştırıldı.

Bulgular: Erken saatlerde EPO'nun lökosit değerlerini artırdığı ($P < 0.05$) ancak zaman geçtikçe etkisinin silikleştiği tespit edilmiştir. Amilaz üzerine de erken saatte artırıcı etki gösterdiği ($P < 0.005$), ilerleyen saatlerde gruplar arasında fark kalmadığını göstermiştir. Serum CRP'si üzerine herhangi bir etkisi ortaya konulamamıştır. Pankreas dokusunda, rakamsal olarak 6.saatte sakrifiye edilen III. grupta ve 6. ve 24. saatlerde incelenen IV. gruplarda orta şiddette pankreatite rastlanmıştır. Sporman skorlamasının rakamsal analizinde anlamlı farklılık olmamasına ($P > 0,05$) karşın, ortaya çıkan orta şiddetteki pankreatit EPO verilen gruplarda oluşmuştur.

Sonuç: Lökosit ve amilaz değerlerinde erken saatlerdeki anlamlı farklılık ilerleyen saatlerde belirsizleşmiştir. Histopatolojik olarak modelin yarattığı şiddetli tablo nedeniyle, daha kontrollü pankreatit tablosunda (düşük oranlı Na taurokolat kullanılan veya ödematöz pankreatit oluşturulan modellerde) EPO'nun etkinliği, ortaya açık olarak konabilir. EPO'nun amilaz ve lökosit üzerine etkisi olduğu ön görülmekle birlikte, hafif pankreatit tablosunda etki, daha net gözlenebilir.

Anahtar Sözcükler: Akut Pankreatit, deneysel model, histopatoloji, eritropoetin

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Introduction

The pancreas is an organ that secretes important enzymes and bioactive amines required for the digestion of carbohydrates, protein and lipids. Under normal conditions, these strong enzymes do not cause any harm to the pancreatic tissue itself. The secretion of such enzymes in inactive forms and the presence of specific storing systems in pancreatic acini and of locally and systemically acting protease inhibitors in the pancreas protect it against these enzymes.

Factors hindering such protective systems result in acute pancreatitis by activating the enzymes and bioactive substances that are in their inactive forms. Clinical and experimental studies have not been able to clearly delineate the etiopathogenesis of acute pancreatitis (1-5).

Each of these responsible factors affects acinar cells in various ways resulting in the retention of potent proteolytic enzymes and their premature activation (3,6). These active enzymes damage acinar cells, which results in rapid secretion of cytokines thereby activating the complement system (7,8). The complement system attracts inflammatory cells like neutrophils and results in their accumulation; neutrophils, on the other hand, are responsible for further secretion of cytokines, free radicals and vasoactive amines like nitric oxide (NO) (9). The inflammatory molecules that are secreted induce pancreatic edema, local effects like necrosis and systemic complications (5,6,10). Cytokines secreted in pancreatitis stimulate apoptosis, which later results in cellular death (3,11,12).

Erythropoietin (EPO) is a member of the cytokine super family and a growth hormone for erythroid lineage. Among its non-erythropoietic effects, it diminishes to a significant degree the edema of inflamed and hypoxic-ischemic tissues and cellular apoptosis, controls inflammation, and lowers tissue lipid peroxidation and generation of malondialdehyde (MDA) (13,14). In clinical conditions like sepsis, in which inflammation cannot be controlled due to the increases in main cytokines like tumor necrosis factor (TNF)-α and interleukin (IL)-β, serum EPO levels have been shown to be suppressed. However, it is not known how the levels of these cytokines or the inflammation itself are affected by the administration of EPO. It has been experimentally demonstrated that NO, the effects of which on the pancreas are controversial, is being suppressed in various ways via administration of EPO (13-15).

In this study we planned to investigate whether the events in the early stages of acute pancreatitis were influenced by EPO and if so, to show to what extent this was a problem. To this end, during the peroperative stage at which acute pancreatitis was triggered or three hours thereafter, erythropoietin was administered. If EPO demonstrated any effect, it was further investigated whether the time of administration was influential. Furthermore, in the presence of an EPO effect, we attempted to histopathologically and biochemically evaluate the changes in the severity of pancreatitis according to different times of sacrifice.

Materials and Methods

60 Wistar Albino rats weighing between 250 – 300 g were used for the experiment. All rats underwent laparotomy under 50 mg/kg ketamine anesthesia (Ketalar®, Eczacıbaşı Warner-Lambert, İstanbul, Turkey).

For inducing acute pancreatitis, the pancreatic canal was approached and 4.5% sodium (Na) taurocholate (0.1 ml / 100 g) (Sigma-Aldrich, Steinheim, Germany) was infused according to retrograde ductal injection model (16-18).

Study protocol (Table 1):

Group I: Sham group (n = 15);

Group II: Control group: acute pancreatitis was induced with the injection of 4.5% Na taurocholate into the pancreatic canal and EPO was not administered (n = 15);

Group III: Study group: acute pancreatitis was induced with the injection of 4.5% Na taurocholate into the pancreatic canal and EPO was simultaneously administered at 400 IU/kg subcutaneously (Eprex®4000 IU/vial Cilag AG, Zug, Switzerland) (n = 15);

Group IV: Acute pancreatitis was induced with the injection of 4.5% Na taurocholate into the pancreatic canal and EPO was administered at 400 IU/kg subcutaneously 3 hours later (Eprex® 4000 IU/vial Cilag AG, Zug, Switzerland) (n = 15).

After 12 hours of fasting, all rats received 50 mg/kg intramuscular ketamine anesthesia. The abdomen was shaved and cleansed with povidone-iodine solution followed by laparotomy. Pancreas and pancreaticobiliary canal were explored and main biliary duct was approached and clipped at its last third portion before

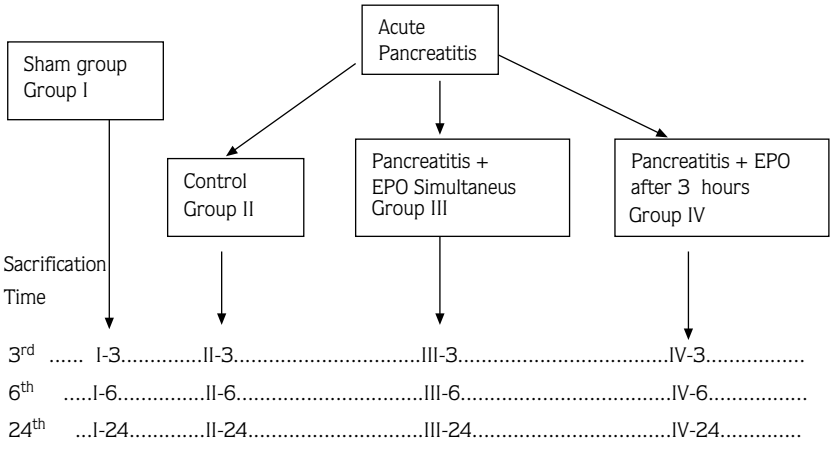

Table 1. Study groups.

joining to pancreatic canal. In this way, it was attempted to eliminate any possible irritating effects of the biliary salt to be administered. The orifice of the pancreaticobiliary duct was passed with a 26 G catheter through transduodenal route; upon visualization of the catheter within the canal, 4.5% Na taurocholate (0.1 ml / 100 g) was manually infused into the pancreatic canal under minimal pressure at a very slow rate. Following the perfusion, the catheter was retrieved; the transduodenal entry hole was sutured with 6/0 round needled prolene to avoid any leakage. Abdominal cavities of all the rats were closed with 3/0 interrupted silk sutures. Immediately after the closure of the abdomen, Group III received SC 400 IU/kg EPO and Group IV received the same dose after three hours.

The groups were further separated into subgroups to be sacrificed at the 3rd, 6th, and 24th hours (n = 5). The rats underwent relaparotomy under 50 mg/kg ketamine anesthesia at the planned (3rd, 6th, 24th) hours. 5 ml of blood was obtained from the right ventricles of all rats with heparin-flushed syringes and the rats were sacrificed with cardiac approach. Blood samples were transferred into 2-3 ml vacutainer tubes for biochemical analysis and 1-2 ml blood was transferred to tubes with EDTA for complete blood count.

Measurement methods

The obtained blood samples were assayed for leukocytes, amylase and C-reactive protein (CRP). Leukocytes were measured (Cell-dyne, Abbott, Santa Clara, USA) with laser reading method; amylase (Olympus AU2700, Mishima, Japan) with enzymatic method; and CRP with immunoturbidimetric technique with the same analyzer.

Histological examination

The pancreatic tissue obtained after sacrifice was separated into five regions to obtain an average of histopathological findings and scoring. Samples were obtained from the following regions (Figure 1):

1. inferior of stomach
2. around the second part of duodenum
3. around the opening of the common pancreaticobiliary canal into the duodenum; head and body of pancreas
4. around the fourth part of duodenum and superior of proximal jejunum
5. he tail of the pancreas adjacent to spleen hilus

The tissue sections were stained with hematoxylin-eosin and were evaluated according to Spormann scores (19,20) (Table 2).

Statistical analysis

For comparison between the groups, one way ANOVA and Tukey post hoc tests were used. For the calculation of p value between repetitive measurements, Student's t test was used.

Means of all the measurements were obtained and are given in the Tables along with standard deviations. A p value of < 0.05 was accepted as having statistical significance.

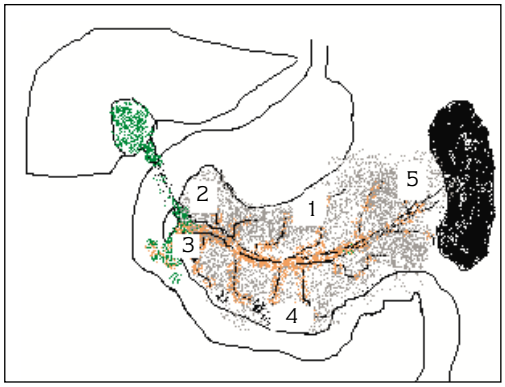

Figure 1. Histopathological samples were obtained from the pancreatic regions.
(1. inferior of stomach, 2. around the second part of duodenum, 3. around the opening of the common pancreaticobiliary canal into the duodenum; head and body of pancreas, 4. around the fourth part of duodenum and superior proximal jejunum, 5. the tail of pancreas adjacent to spleen hilus).

Table 2. Spormann scores (19,20).

Findings	Severity	Score
Edema	Mild	1
	Moderate	2
	Severe	3
İnflammation	Mild	1
	Moderate	2
	Severe	3
Fat necrosis	< 2	3
	3-5	5
	< 5	7
Parenchymal necrosis	Mild	3
	Moderate	5
	Severe	7
Hemorrhage	Mild	3
	Moderate	5
	Severe	7

Results

Biochemical Evaluation

Leukocyte levels

Among the groups sacrificed at 3 hours, leukocyte levels were highest in group III and this was more significant than in other groups ($P < 0.005$).

At 6 hours, leukocyte levels were lowest in group II with pancreatitis ($P < 0.05$). In Group IV that had EPO effect for 3 hours, leukocyte levels were higher than those of group III ($P < 0.001$). Leukocyte levels of all groups were similar at 24 hours (Table 3).

Table 3. Mean leukocyte levels (SD) of the groups.

Groups	3rd hour	6th hour	24th hour
I	2880.0 (460.4)	5588.0 (179.0)	2826.7 (176.0)
II	10270.0 (733.0)	8770.0 (264.0)	4052.0 (387.0)
III	11900.0 (215.0)	9878.0 (311.0)	4417.0 (852.0)
IV	10848.0 (174.5)	11970.0 (345.3)	4246.0 (265.5)

When different times of sacrifice were compared, it was seen that the leukocyte levels that were highest at 3 hours decreased considerably thereafter (p: 0.005).

Serum amylase measurements

Among the groups analyzed at 3 hours, amylase levels were highest in group III ($P < 0.005$). Mean amylase level of group IV was close to this value ($P > 0.05$).

Although the parameters of all the groups were close in value by 6 hours, group II levels were higher ($P > 0.05$).

Mean values at 24 hours were similar to mean values at 6 hours ($P > 0.05$) (Table 4).

Table 4. Mean amylase levels (SD) of the groups.

Groups	3rd hour	6th hour	24th hour
I	454.8 (15.5)	676.6 (18.0)	288.4 (38.0)
II	2209.6 (288.0)	3922.2 (441.0)	3948.0 (347.6)
III	3019.2 (441.0)	3629.0 (597.0)	3820.0 (355.0)
IV	2368.0 (254.0)	3250.0 (314.0)	3494.0 (441.0)

Evaluation of different hours of sacrifice revealed that amylase levels increased over time ($P < 0.05$).

Serum CRP levels

No group other than the laparotomy group had significantly different levels of mean CRP ($P > 0.05$) (Table 5). At different hours of sacrifice in the same groups, CRP showed insignificant decreases from the 3rd to 6th hours and showed significant increases at 24 hours ($P \leq 0.002$).

Table 5. Mean CRP levels (SD) of the groups.

Groups	3rd hour	6th hour	24th hour
I	22.2 (2.7)	18.2 (1.5)	29.2 (1.6)
II	33.2 (2.0)	27.4 (1.7)	63.2 (6.0)
III	33.2 (3.6)	26.8 (3.6)	65.2 (7.0)
IV	33.2 (2.0)	27.4 (1.1)	64.8 (5.8)

Histopathological Examination

The tissues obtained from the pancreas were separated as explained above and were evaluated according to Spormann scores (19,20) (Table 6). The highest score was 27, correlating with the most severe form of pancreatitis. This score was divided by three and further classification was as follows: Mild ≤11, moderate: 11-19, and severe pancreatitis: 19-27.

Table 6. Spormann score (SD) of the groups.

Groups	3rd hour	6th hour	24th hour
I	6.0 (0.3)	5.8 (0.3)	5.9 (0.4)
II	19.5 (1.2)	19.4 (0.9)	20.3 (0.7)
III	19.7 (0.4)	17.9 (1.0)	19.9 (0.7)
IV	19.4 (1.0)	16.2 (0.5)	17.9 (0.9)

The pancreatic tissue of the laparotomy group corresponded to the mildest degree of pancreatitis among all groups ($P \leq 0.002$) (Table 7). In group III sacrificed at 6 hours and group IV sacrificed at 6 and 24 hours, pancreatitis was moderate; other groups had severe pancreatitis. However, none of the parameters in the Spormann scoring criteria was different or significant. Furthermore, groups administered EPO showed similarities.

Table 7. Pancreatitis classification of all groups according to Spormann scores.

Groups	3rd hour	6th hour	24th hour
I	Mild	Mild	Mild
II	Severe	Severe	Severe
III	Severe	Moderate	Severe
IV	Severe	Moderate	Moderate

Discussion

As animal models of retrograde injection/perfusion of bile salts (Na taurocholate) into the pancreatic canal had similar etiological factors with that of humans, while resulting in similar local and remote effects and making it possible to investigate different therapeutic modalities, this was chosen as the model in our study (21). An experimental model of acute pancreatitis has been successfully created as shown by biochemical and histopathological examinations.

While EPO increased leukocytosis in the early hours, this effect disappeared in time resulting in a decrease in leukocytes at 24 hours. EPO also caused an increase in amylase levels in the early hours, yet this effect that created a difference among the groups was short-lived, and was not observed in the following hours. The severity of the pancreatitis seen in this model might have masked the influence of EPO on amylase levels.

CRP is a marker dictating the severity of acute pancreatitis, with a value between the 48th-72nd hours (22). In our study groups CRP levels remained unchanged until 24 hours and started increasing thereafter, and this finding is consistent with the information in the literature. Interestingly, there was an insignificant decrease between the 3rd to 6th hours. This slight decrease in early hours might be similar to the changes in IL-6 (9), since it is well known that CRP correlates with increases in IL-6 (3,23), though CRP makes a later peak in comparison (24). Thus, CRP has lower sensitivity and specificity during the early stages of inflammation (4,6).

The increase in the levels of serum calcium or the cytosolic calcium in the acinar cells is known to be one of the initiating events for acute pancreatitis (25). Increasing levels of cytosolic calcium result in the activation of zymogen granules, which play an important role in early stages of pancreatitis (26). Several research studies have shown that for a rapid and limited period of time, EPO increases the intracellular cytosolic [Ca^{++}] concentration, thus increasing cellular excitability (13,14,27-29).

EPO is identified to have receptors in different tissues (30,31). However, there is no data regarding the presence of its receptors in the pancreas. In the event of EPO receptors in the pancreas, we might anticipate an increase in the severity of pancreatitis together with an increase in the concentrations of cytosolic calcium.

However, EPO did not aggravate pancreatitis in this study.

Despite being insignificant, EPO resulted in an increase in amylasemia in this study. The group administered EPO and the pancreatitis group did not differ regarding pancreatic edema.

In smooth muscle cell cultures, Kusano et al. (32) demonstrated that EPO prevented the productions of NO and cyclic guanosine monophosphate (cGMP) as stimulated by IL-1β, thereby regulating cellular apoptosis. Excessive production of NO as a result of the stimulation of cytokines can be prevented with EPO administration; therefore, hypotensive effects of NO can be reversed with EPO (32).

High levels of cytokines in the body aggravate a variety of disease states. Gene rally, the presence of cytokines in the body or increases thereof (in conditions like inflammation or sepsis) reduce the concentrations of EPO (33,34). However, Krafte-Jacobs and Bock (35) identified increases in EPO together with IL-6 in circulations of children with sepsis or septic shock.

Activation of peritoneal macrophages is known to have a role in acute pancreatitis. Rendering of peritoneal macrophages passive by EPO will at least eliminate the contribution of these cells to the severity of acute pancreatitis. However, in this study neuronal ischemic damage induced by the generation of NO was prevented by EPO either by decreasing free radicals generated via NO or by antagonizing their toxicities (15). In a brain ischemia model in rats, EPO was administered immediately after the injury; thereby excessive production of NO, which induced ischemic injury, was suppressed, levels of post-ischemic MDA, brain edema and neuronal loss were all reduced and survival was lengthened (36).

We still do not know what type of an effect EPO exerts in the tissue in case of acute pancreatitis. This study demonstrated that EPO increased the levels of leukocytes in the early hours but the difference disappeared in time. It had an increasing effect on amylase within the first 3 hours and caused insignificant decreases at 6 and 24 hours. It is possible that in the presence of edematous pancreatitis, the influence of EPO on amylase can be more clearly demonstrated. No effect could be demonstrated on serum CRP. The laparotomy group had mild pancreatitis due to surgical trauma and minimal manipulation. There was moderate degree of pancreatitis at 6 hours in group III and at 6 and 24 hours in group IV. In the numerical analysis of Spormann scoring, there was no significant difference; however, moderate pancreatitis was present in groups administered EPO. Edema, inflammation, fat necrosis, parenchymal necrosis and hemorrhage were the parameters investigated by Spormann scoring and none of them showed any difference. The severity of the pancreatitis induced prevented satisfactory evaluation of the mentioned parameters.

The results we have obtained demonstrate that more controlled models (employing low rates of sodium taurocholate or edematous pancreatitis) are necessary to better investigate the efficacy of EPO.

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