

Early Treatment with Metformin Decreases Pancreatic Damage in Rats with LPS Induced Sepsis

LPS ile Oluşturulan Sepsisli Sıçanlarda Metformin ile Erken Tedavi, Pankreatik Hasarı Azaltır

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Abstract

In this study, the effects of metformin on pancreatic tissue after lipopolysaccharide (LPS)-induced sepsis were investigated. 30 Sprague Dawley male rats were used in the study. Five groups were formed: control, sepsis, sepsis+1 hour before metformin, sepsis+1 hour later metformin and sepsis+3 hour later metformin as 6 animals in each group. LPS and metformin was prepared at 5 mg/kg and 200 mg/kg volumes, respectively, and injected intraperitoneally to the rats. Blood samples and pancreas tissues were taken from the rats 24 hours after LPS injection. Amylase, glucose and insulin parameters were measured in serum of rats. Malondialdehyde (MDA) and myeloperoxidase (MPO) parameters in pancreas tissues of rats were evaluated. Pancreatic tissues were examined by hematoxylin-eosin (H-E) staining method histopathologically. When the results were evaluated, it was seen that LPS caused sepsis and pancreatic tissue damage in rats. However, it has been determined that metformin significantly alleviates these damages in the treatment groups. In particular, metformin administered prior to sepsis has been shown to have protective effects in the pancreatic tissues of rats.

Key words: Lipopolysaccharide, Metformin, Pancreas, Sepsis, Rat.

Öz

Bu çalışmada, metforminin lipopolisakkarit (LPS) ile indüklenen sepsis sonrası pankreas dokusu üzerindeki etkilerinin araştırılması amaçlanmıştır. Çalışmada 30 adet Sprague Dawley cinsi erkek sıçan kullanılmıştır. Gruplar 5 adet ve her grupta 6 tane hayvan olacak şekilde; kontrol, sepsis, sepsis+1 saat önce metformin, sepsis+1 saat sonra metformin ve sepsis+3 saat sonra metformin olarak belirlenmiştir. LPS ve metformin dozları sırası ile 5 mg/kg ve 200 mg/kg olarak belirlenmiş olup hayvanlara intraperitoneal olarak uygulanmıştır. LPS enjeksiyonundan 24 saat sonra hayvanların kan örnekleri ve pankreas dokuları cerrahi işlemlerle alınmıştır. Sıçanların serumlarında amilaz, insülin ve glukoz düzeyleri ölçülmüştür. Alınan pankreas dokularında ise malondialdehit (MDA) ve myeloperoksidaz (MPO) düzeyleri ölçülmüştür. Ayrıca pankreas dokuları hematoksin-eosin (H-E) boyama yöntemi ile histopatolojik olarak değerlendirilmiştir. Sonuçlar incelendiğinde, LPS'nin sıçanlarda sepsis meydana getirdiği ve pankreas dokularında hasar oluşturduğu görülmüştür. Ancak, metforminin tedavi gruplarında bu hasarı önemli ölçüde hafiflettiği tespit edilmiştir. Özellikle, sıçanlara sepsis oluşturulmadan önce uygulanan metforminin daha etkili olduğu, pankreas dokularında koruyucu etkilere sahip olduğu gösterilmiştir.

Anahtar kelimeler: Lipopolisakkarit, Metformin, Pankreas, Sepsis, Sıçan.

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1. Introduction

Systemic inflammatory response (SIRS) due to an infection is defined as sepsis. Sepsis is the result of bacterial infections with some clinical signs in the body. These findings include increased leukocyte count, hyperthermia, tachycardia, tachypnea etc. symptoms (Cai et al. 2010). Sepsis is an expensive medical problem affecting the whole world and is the primary cause of mortality in intensive care units (Bosmann and Ward 2013).

In the pathophysiology of sepsis, pathogens and inflammatory responses are present. Research has shown that humoral system is active in the body and that various cytokines are released as a result of infection and traumatic damage in tissues (Bone 1991; Nathan 2002). SIRS can trigger with many different factors. For example, infectious agents such as lipopolysaccharide released from bacteria or non-infectious damaged cells released

after ischemia-reperfusion or trauma (Erridge 2011). The Toll-like receptor (TLR)-4 signaling pathway leading to the production of pro-inflammatory mediators has been proposed as a key pathway in the pathophysiology of sepsis (Bosmann and Ward 2013). These receptors, which recognize the infection, activate macrophages and mast cells and initiate a variety of inflammatory processes mediated by them, including chemokines, cytokines, vasoactive amines, eicosanoids and proteolytic cascade mechanics (Medzhitov 2008).

Sepsis is most often caused by toxins of Gram-positive and Gram-negative bacteria. Viruses, fungi and protozoa can also develop secondary, although rarely. Because of the more frequent effects of Gram negative bacteria, Gram negative bacterial toxins are used especially in studies done by sepsis pathogenesis (Bosmann and Ward 2013).

Lipopolysaccharide (LPS), an amphiphilic member in the outer membrane of Gram-negative bacteria, performs two major functions for bacteria. The high concentration in the outer membrane reflects the function of protecting the integrity of the cell membrane and of mediating the bacterial interaction with the environment (Steimle et al. 2016). The endotoxicity of LPS is due to its chemical structure. The polysaccharide moiety in the structure consists of the ring called O-antigen and is responsible for the specificity of the endotoxin. The other region, the core chain part, is the same as the general structure in Gram-negative bacteria. The lipid region (lipid-A) in the phospholipid structure is the main structure which causes toxicity and inflammation (Raetz and Whitfield 2002).

Metformin is considered an anti-hyperglycemic agent because it reduces the blood sugar concentration in Type II Diabetes Mellitus without significant hypoglycemia. Metformin also causes a marked decrease in insulin resistance and fasting insulin levels in the plasma (Viollet et al. 2012). It primarily lowers plasma glucose levels through the suppression of hepatic glucose production as a consequence of the reduction in gluconeogenesis (Stumvoll et al. 1995). However, besides all these effects, metformin has also been shown to have anti-inflammatory effects and to protect the pancreas from inflammatory complications caused by diabetes at the same time (Saisho 2015; Murad et al. 2015; Jiang et al. 2017).

In this study, it was aimed to investigate the role of metformin in acute sepsis in rats induced by LPS and determined its protective or therapeutic effects on pancreatic tissue. It is aimed to measure the effects of LPS on pancreatic tissue by histopathological and biochemical parameters and to determine the effect of metformin on these influences.

2. Materials and Methods

2.1 Animal Care and Operations

Ethical committee approval was obtained from Eskişehir Osmangazi University before the experimental procedures were started (545/2016). Thirty male Sprague-Dawley rats (250–300 g body weight) were used for the study. Animals were obtained from Eskişehir Osmangazi University Medical and Surgical Experimental Animals Application

and Research Center (TICAM). The rats were maintained at $22 \pm 2.0^\circ\text{C}$ and $50\% \pm 5\%$ humidified environment. Rats had access to food and water *ad libitum*. LPS and metformin solutions were prepared with saline and these solutions were injected intraperitoneally to rats. Doses of metformin and LPS were determined as 200 mg/kg and 5 mg/kg, respectively, as a result of literature searches (Cho et al. 2009; Quaile et al. 2010).

The rats were divided into five groups: control, sepsis, pre-sepsis metformin, sepsis+1 metformin and sepsis+3 metformin groups. The control group rats were injected with saline. Rats in the sepsis group were injected with LPS. In the pre-sepsis metformin group, metformin was injected one hour before LPS injection. In the Sepsis+1 metformin group, metformin injection was performed one hour after LPS injection. Similarly, in the Sepsis+3 metformin group, metformin injection was performed three hours after LPS injection. All animals were sacrificed 24 hours after LPS injection.

2.2 Collection of Blood and Tissue Samples

The animals were sacrificed by taking blood by cardiac puncture under intramuscular anesthesia with ketamine (80 mg/kg) and xylazine (10 mg/kg). Blood samples taken in red blood tubes were centrifuged at 3500 rpm for 15 min to obtain serum. For histopathological and biochemical measurements pancreas tissues of animals were obtained. Examples to be used in histology were placed in 10% formaldehyde. All serum and tissue samples were stored at -80°C until biochemical measurements were analyzed.

2.3 Measurements in Serum Samples

Serum amylase, insulin and glucose levels were determined in the Roche Cobas c501 auto analyzer device (Roche Diagnostic GmbH, Mannheim).

2.4 Malondialdehyde (MDA) Assay

The MDA measurement in pancreas tissues was made according to the method of Ohkawa et al. (1979). Approximately 100 mg of tissues were homogenized in 1000 μL of ice-cold 1.15% potassium chloride (KCl) solution. After homogenization, samples were centrifuged at 4°C 1000 rpm for 15 minutes and supernatants were removed. Supernatants were incubated by adding Sodium dodecyl sulfate (SDS), Acetate buffer and Thiobarbituric acid (TBA) for 60 minutes at 95°C . The absorbance of the samples that were centrifuged again at 4000 rpm after the incubation, was measured spectrophotometrically at 532 nm.

2.5 Myeloperoxidase (MPO) Assay

Myeloperoxidase activity was measured spectrophotometrically by kinetic method, as determined by Suzuki et al. (1983). One hundred milligrams of pancreas tissue was homogenized in 50 mM phosphate buffer (pH 7.4) and centrifuged at 4°C for 5 minutes at 7000 rpm. The remaining pellets were homogenized again with cold Phosphate buffer (50 mM, pH 6.0) which

containing 0.5% Hexadecyltrimethylammonium bromide (HETAB) and 10 mM Ethylenediaminetetraacetic Acid (EDTA). Phosphate buffer (50 mM, pH 5.4) and Tetramethylbenzidine (TMB) (dissolved in DMF) were added to the homogenized samples. Samples were recorded for absorbance changes at 655 nm for 3 minutes by adding H₂O₂ immediately prior to absorbance measurement.

2.6 Total Protein Assay

Total protein levels in pancreatic tissues were determined according to the Biuret measurement method determined by Gornall et al. (1949). Briefly, under alkaline conditions, Cu²⁺ ions form a blue-violet complex with ammonium compounds such as peptides and proteins. The absorbances of colored complex products are measured by spectrophotometry at 545 nm wavelength. MDA and MPO measurement results are given as a ratio to total protein levels.

2.7 Histopathological Analyzes

At the end of the experiment, the pancreases of the sacrificed rats were carefully removed and then fixed in 10% buffered formaldehyde for 24 hours. After rinsing under tap water, dehydration was carried out by passing the grades through increasing ethyl alcohols. Then, xylene was clarified in the liquid paraffin and then put into paraffin blocks. Serial sections of 5 µm thickness were taken from the blocks and painted with H-E. Histopathological examination was performed under a binocular microscope to obtain micro-images representing the group.

2.8 Statistical Analysis

Normal distributions of all parameters were examined by Kolmogorov-Smirnov and Shapiro-Wilk tests. The One Way ANOVA test was used for the independent and normal variables. Probability values of p<0.05 were considered significant. All data analyzes were done with IBM SPSS Statistics 21 package programs.

3. Results

Our results are summarized statistically by Tab. 1 and Figs. 1–3. When amylase and glucose levels were examined, there was a significant increase in sepsis and post metformin groups according to the control group (p<0.001) (Tab. 1). However, when compared with the sepsis group, it was observed that there was a significant decrease in all treatment groups (p<0.001). Significant differences were observed between pre-sepsis metformin group and post-sepsis metformin groups (p<0.001). LPS administration significantly reduced insulin levels in rats (p<0.001) (Tab. 1). However, metformin administration increased insulin levels at a significant level, especially in groups administered one hour before and one hour later (p<0.001). In the group that received metformin three hours later, no significant increase occurred.

When the levels of MDA were examined, significant increase was observed in rats after LPS administration (p<0.001) (Fig. 1). However, in the treatment groups given metformin, the MDA levels decreased significantly in comparison with the rats in the sepsis group (p<0.001). In the group that received metformin three hours later, the levels were higher than the pre-sepsis metformin group.

Table 1. Effects of metformin on serum amylase, insulin and glucose levels in the rats (mean ± SD).

Groups	N	Amylase (U/L)	Insulin (µU/mL)	Glucose (mg/dL)
Control	6	885.000 ± 45.135 ^b	15.463 ± 1.410 ^b	112.167 ± 24.227 ^b
Sepsis	6	2967.167 ± 317.609 ^a	3.673 ± 1.059 ^a	415.667 ± 31.067 ^a
Pre-sepsis metformin	6	1197.167 ± 101.618 ^b	9.865 ± 1.279 ^{a,b}	168.500 ± 25.712 ^b
Sepsis+1 metformin	6	1443.333 ± 80.042 ^{a,b,c}	7.527 ± 0.565 ^{a,b}	245.667 ± 23.830 ^{a,b,c}
Sepsis+3 metformin	6	1759.667 ± 63.254 ^{a,b,c}	6.323 ± 0.669 ^{a,c}	307.333 ± 33.140 ^{a,b,c}

^a p<0.001 compared with control group; ^b p<0.001 compared with sepsis group; ^c p<0.001 compared with pre-sepsis metformin group.

LPS administered to rats significantly increased pancreatic MPO levels in sepsis group (p<0.001) (Fig. 2). MPO activity in pre-sepsis metformin and sepsis+1 metformin groups decreased significantly (p<0.001). When we looked at treatment groups, MPO activity in sepsis+3 metformin group was significantly higher than control.

Histopathological studies have shown that LPS causes only slight changes and edema in the pancreatic islet cells and Langerhans islets (Fig. 3). It was observed that administration of metformin 1 hour before or 1 hour after LPS relieved these changes but administration of metformin 3 hours after LPS resulted in similar changes to the sepsis group.

4. Discussion

The pathophysiology of sepsis, leading to systemic inflammatory response syndrome (SIRS), multiple organ failure and death, is one of the most important and costly mortality causes worldwide. Today, sepsis, which still has no definitive treatment, maintains its update among the deadly diseases as a serious medical problem (Bosmann and Ward 2013). One of the leading causes of sepsis is Gram-negative bacterial endotoxin LPS has recently been shown to be intestinally absorbed in the organism leading to systemic inflammation and pancreatic damage (Ramakrishna 2013; Bradlow 2014; Zhou et al. 2016).

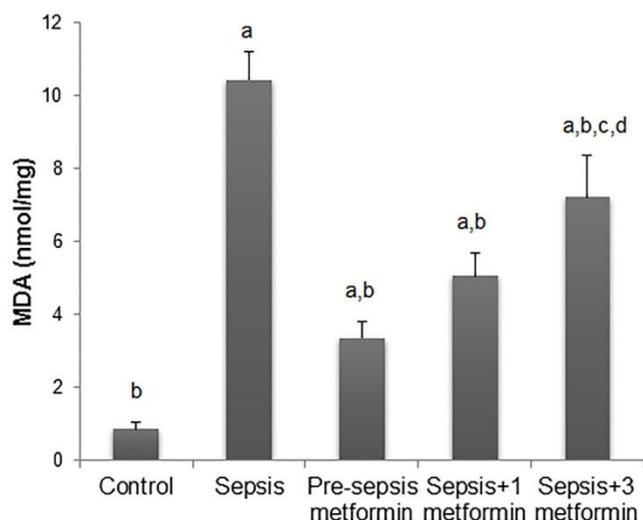


Figure 1. Effects of metformin on pancreatic tissue's MDA levels in the rats (mean \pm SD).

^a $p < 0.001$ compared with control group; ^b $p < 0.001$ compared with sepsis group; ^c $p < 0.001$ compared with pre-sepsis metformin group; ^d $p < 0.001$ compared with sepsis+1 metformin group.

In the light of the literature studies, we aimed to investigate the effects of sepsis caused by LPS on rat pancreas in our study. When our results of sepsis group were assessed, biochemical parameters were shown to be associated with systemic sepsis and pancreatic damage in rats with LPS. Histologically, there is no significant severe damage in the pancreatic tissues, but our results are consistent with the literature (Ding et al. 2003; Tian et al. 2013). Since the dose of LPS used is low, it may not have caused excessive damage to the rats. In our study, we used metformin in treatment groups. Metformin has proven to be not only a powerful anti-hyperglycemic agent but also a drug with anti-inflammatory properties (Kim and Choi 2012; Salman et al. 2013; Pandey and Kumar 2016). In addition, studies have shown that infections and inflammatory mechanisms play a role in the development of diabetes (Jiang et al. 2017). For this reason, we aimed to see the effects of metformin on the pancreas as a result of damage after sepsis.

Insulin, amylase and glucose levels were measured in the serum of rats to demonstrate cellular damage of the pancreas (Murad et al. 2015). When the results were evaluated, all values of the rats in the groups given metformin improved. When statistically assessed, the most significant results were obtained in the pre-sepsis metformin group.

Since oxidative stress is influenced in the case of inflammation, MDA levels that are the most commonly measured as clinically indicators of oxidative damage have been evaluated (Houstis et al. 2006; Zhou et al. 2016). Sepsis-induced inflammation was demonstrated by MPO activity, a sign of neutrophil infiltration (Klebanoff 1999). As a result of our study, increased levels of MDA and MPO in the sepsis group decreased significantly with metformin. The most significant decrease was found in pre-sepsis metformin group but these levels in sepsis+3 metformin group remained high.

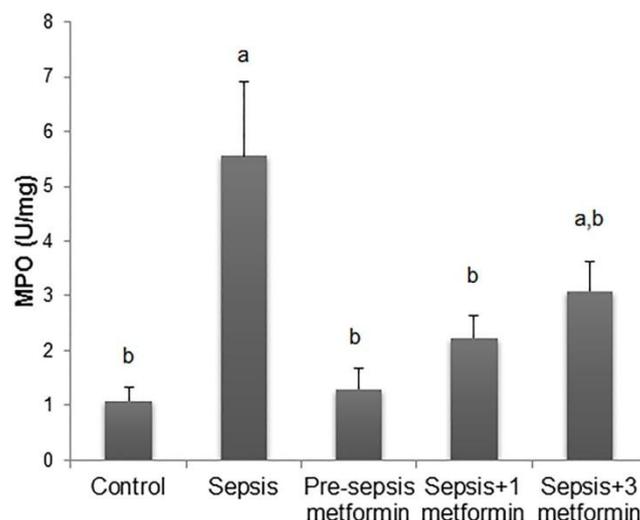


Figure 2. Effects of metformin on pancreatic tissue's MPO activities in the rats (mean \pm SD).

^a $p < 0.001$ compared with control group; ^b $p < 0.001$ compared with sepsis group.

Both the biochemical parameters and the results of histological examination in the pre-sepsis metformin group are close to the control group, suggesting that metformin provides a significant protective effect against sepsis and oxidative stress that occurred in the pancreas. Despite relatively improved values in the sepsis+1 metformin group, histological and biochemical findings, especially in the sepsis+3 metformin group, close to the sepsis group indicate that metformin is inadequate to treat after inflammation has occurred.

5. Conclusion

In conclusion, it is clear that the effect of metformin on endotoxemia attenuation of LPS and the improvement of pancreatic damage. However, although there are relatively improved values in the post-sepsis metformin, it can be interpreted that metformin is a preventative but not therapeutic treatment of sepsis, since administration before sepsis gives the closest results to the control group. For this reason, metformin may become a protective agent for patients who are at risk of sepsis. In terms of increasing the reliability of our results, it may be useful to develop different studies under different conditions and doses.

Conflicts of Interest: No conflict of interest was declared by the authors.

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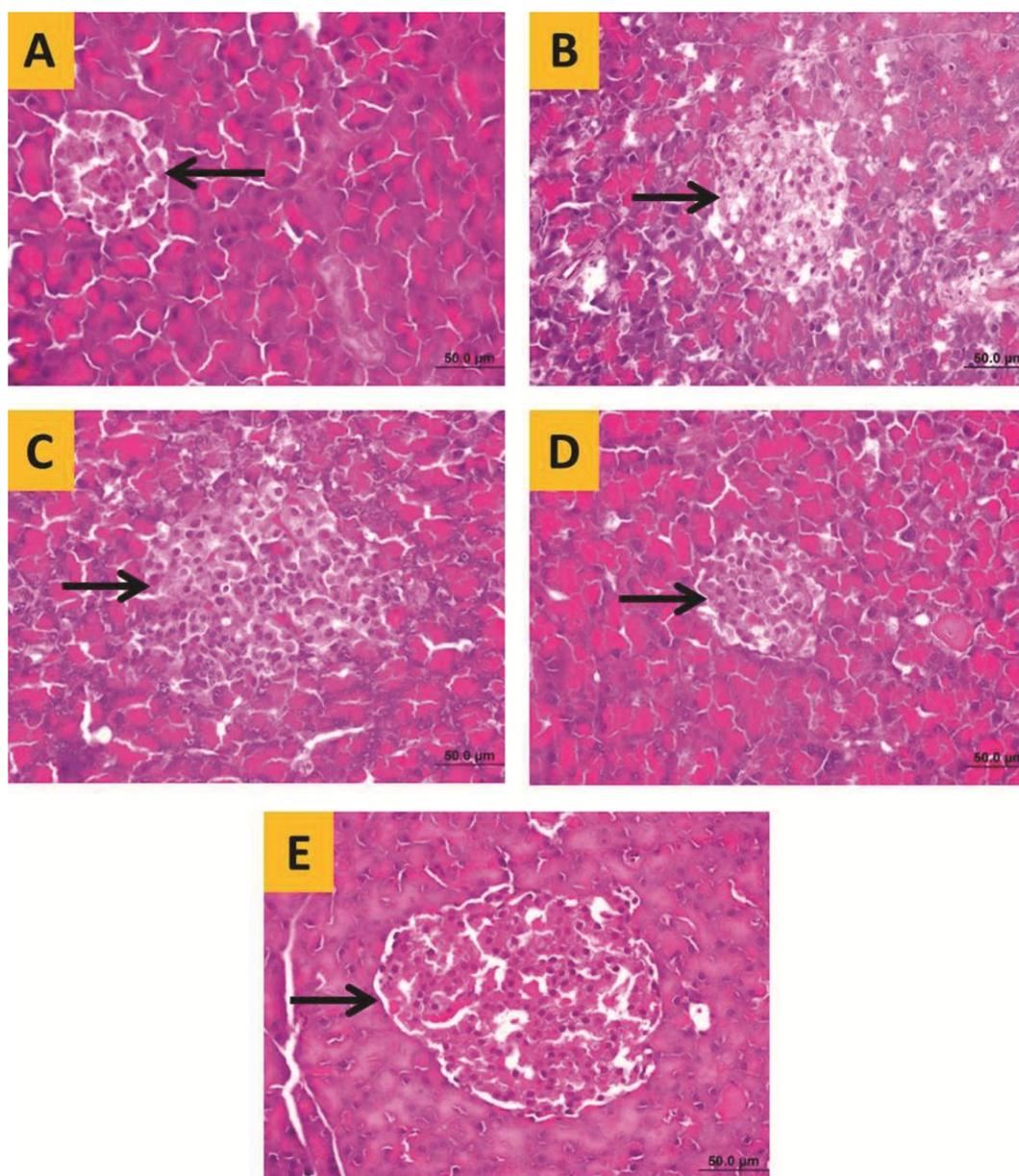


Figure 3. Histological views of pancreatic tissues. The arrows show the islets of Langerhans. **A:** Acinar cells and Langerhans islets in the control group are typical histologic appearance. **B:** It is noteworthy that in the group given only LPS, cells are separated due to degenerative changes and mild edema in some acinar and Langerhans islet cells. **C:** Histological appearance is normal in metformin treated group 1 hour before LPS. **D:** Typical histological appearance is observed in the group treated with metformin 1 hour after LPS. **E:** In metformin given 3 hours after LPS group seems to have edema in some Langerhans islets. All sections were stained with H-E. The bars are all 50 µm.

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