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Evaluation of effects of bupivacaine and isoflurane on pancreas damage after renal ischemia-reperfusion injury: An experimental study

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Abstract. *Recent studies have shown that renal ischemia-reperfusion injury can have detrimental effects on distant organs such as the brain, liver and lungs. In this study, we aimed to investigate the effects of renal ischemia-reperfusion injury on pancreatic functions.*

Materials and Methods. Twenty four male adult Wistar rats were divided into four groups. Sham and control group animals were not given any medications. Animals in groups 3 and 4 were treated with epidural bupivacaine and isoflurane inhalation. Animals in all groups except for the sham group were subjected to bilateral renal ischemia for 45 minutes and subsequent reperfusion. Blood samples were collected before ischemia, immediately after reperfusion and 2h after reperfusion. Serum blood urea nitrogen, creatinine, amylase and lipase levels were measured, and pancreatic sections were histopathologically examined for the presence and severity of congestion, degenerative cellular changes, cytoplasmic vacuolization and leukocytic infiltration. Levels of malondialdehyde, endogenous antioxidant enzyme catalase and reduced glutathione were measured in pancreatic tissue sections by using colorimetric kits.

Results. Serum blood urea nitrogen and creatinine levels increased in rats subjected to renal ischemia-reperfusion. There was no difference between the groups in terms of pancreatic tissue malondialdehyde, catalase and glutathione levels.

Conclusion. In conclusion, bilateral renal ischemia for 45 minutes led to significant impairment in pancreatic function and changes in pancreas histology. These findings might be due to antioxidant deficiency and increased lipid peroxidation in pancreatic tissue.

Key Words: *Ischemia-reperfusion, bupivacaine, pancreas damage.*

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Оцінка впливу бупівакаїну та ізофлурану на пошкодження підшлункової залози після ішемічно-реперфузійного гострого пошкодження нирок: Експериментальне дослідження

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Резюме. *Недавні дослідження показали, що ішемічно-реперфузійне пошкодження нирок може мати негативний вплив на мозок, печінку та легені. У цьому дослідженні ми мали на меті дослідити наслідки ішемічно-реперфузійного гострого пошкодження нирок на функції підшлункової залози.*

Методи. Двадцять чотири самці щурів Вістар були розділені на чотири групи. Щурам симуляційної (група без втручання «sham group») та контрольної груп не давали ніяких ліків. Тварин 3 та 4 груп лікували епідуральним бупівакаїном та ізофлураном для інгаляцій. Тварин у всіх групах, за винятком симуляційної, піддавали двобічній ішемії нирок протягом 45 хвилин та подальшій реперфузії. Зразки крові відбирали до ішемії, відразу після реперфузії та через 2 години після реперфузії. Вимірювали рівні сечовини, креатиніну, амілази та ліпази; гістопатологічно досліджували зрізи підшлункової залози на наявність та тяжкість застійних явищ, дегенеративних клітинних змін, вакуолізації цитоплазми та лейкоцитарної інфільтрації. Рівні малонового діальдегіду, ендогенного антиоксидантного ферменту каталази та глутатіону вимірювали на зрізах тканин підшлункової залози за допомогою колориметричних наборів.

Результати. Концентрації сечовини та креатиніну у сироватці крові підвищувались у щурів, які піддавались ішемічній-реперфузії нирок. Між групами не було різниці щодо рівнів малонового діальдегіду, каталази та глутатіону у зразках тканин підшлункової залози.

Висновок. Двобічна ішемія нирок протягом 45 хвилин призводила до значного порушення функції підшлункової залози та зміни гістології підшлункової залози.

Ключові слова: *ішемія-реперфузія, бупівакаїн, пошкодження підшлункової залози.*

Introduction. Renal ischemia-reperfusion (I/R) injury inevitably occurs in procedures such as kidney transplantation, partial nephrectomy and renovascular surgeries during which renal arterial blood inflow is temporarily interrupted [1]. It is a significant cause of acute kidney injury [2]. Ischemia initiates the injury by deprivation of the energy needed to maintain ionic gradients and homeostasis, which may ultimately lead to cellular dysfunction and death. At the same time, reperfusion exacerbates this damage by triggering an inflammatory reaction via free radicals, endothelial factors and leukocytes [1, 2]. Reactive oxygen species (ROS) cause cell damage during I/R injury, and an imbalance between the production of ROS and antioxidant capacity of the target cells is known to have an essential role in this process [1].

Millions of patients worldwide are exposed to inhalation anesthesia annually. In addition to their anesthetic effects, volatile anesthetics also have non-anesthetic physiological effects. There are studies about the protective effects of volatile anesthetics in the literature [3]. There is evidence that volatile anesthetics are protective against I/R injury in heart and lungs. Specifically, pretreatment with volatile anesthetics before cardiac ischemia protects against I/R injury [4]. However, mechanisms by which these medications protect the organs is unclear. In some studies, it has been suggested that volatile anesthetics protect the heart via activation of adenosine triphosphate-dependent potassium channels [5]. On the other hand, some studies reported that volatile anesthetics protect the heart and lungs against I/R injury via anti-inflammatory mechanisms [6].

Renal I/R elicits tissue damage in several organs: heart, lung and liver [1]. The I/R injury, which occurs in the pancreas via xanthine oxidase enzyme, can affect renal function. However, there are not sufficient data regarding the effects of renal I/R on the pancreas [7].

In this study, we aimed to analyze the potentially detrimental effects of renal I/R injury on the pancreas by focusing on both pancreatic function and histology.

Materials and Methods. The ethical committee of Ahi Evran University approved this study (2016-16/1). Twenty-four male Wistar rats weighing between 200 and 250 gr were used. Animals were bred and housed

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with an altering 12-hour light and dark cycle in metal cages at standard room temperature (20°C) and fed by standard laboratory chow. They had free access to tap water and chow except for the pre-operative fasting period of 2 hours. Their body temperatures were monitored by rectal thermometers and maintained at 36 to 37°C. The rats were anesthetized with intramuscular ketamine, and the tail vein was cannulated for fluid or drug administration. The rats were divided into four groups:

Group 1: Sham group

Group 2: Control group animals that were subjected to renal I/ R injury and were not treated with any medications

Group 3: Animals subjected to renal I/R and treated with bupivacaine

Group 4: Animals subjected to renal I/R and treated with isoflurane inhalation

Rats in groups 1 and 2 were anesthetized with ketamine, and any other anesthetic agents were not given. Rats in group 1 were subjected to midline laparotomy only while the rats in group 2 were subjected to subsequent renal I/R. All these rats were kept in closed cages on spontaneous breathing with 100% oxygen after surgery.

In group 3, rats were anesthetized with ketamine, and an epidural catheter was placed through the intervertebral space between L3 and L4 and advanced to the T6 level. Bupivacaine (0,5%) infusion was performed through this catheter at the rate of 20 μL/h for 2 hours. They were then put into closed cages and remained on spontaneous breathing with 100% oxygen.

In group 4, rats that were anesthetized with intramuscular ketamine and kept on spontaneous breathing in closed cages were exposed to 1,2% isoflurane mixed with 100% oxygen by using an agent-specific vaporizer for 2 hours after reperfusion.

Blood samples were collected before ischemia, immediately after reperfusion and 2h after reperfusion. After prepping, a midline laparotomy was performed, and both renal pedicles were exposed transperitoneal. Renal arteries were identified and clamped for 45 minutes using vascular Bulldog clamps at both sides to create ischemia. Subsequently, the clamps were removed, and reperfusion was initiated. Both kidneys were inspected for 1 minute following clamp removal to ensure restoration of blood inflow by changes in the color of renal parenchymas from purple to pink. Subsequently, abdominal wall and skin of the rats were closed. Blood samples were collected 2 hours after reperfusion from all animals, which were subsequently sacrificed. Pancreata of these animals were removed immediately after sacrification. Blood urea nitrogen (BUN), creatinine (Cr), amylase and lipase levels were measured in blood samples collected after I/R and pancreatic tissue sections were histopathologically assessed for presence and severity of congestion, degenerative cellular changes, cytoplasmic vacuolization and leukocytic infiltration.

The enzyme CAT converts hydrogen peroxide (H_2O_2) to oxygen (O_2) and water (H2O). In oxidative stress, level of the enzyme superoxide dismutase (SOD) increases to counteract and scavenge superoxide anions (O^{2-}) while activity of the CAT enzyme decreases [8]. The SOD enzyme converts superoxide to hydrogen peroxide and oxygen. Glutathione (GSH) is the primary non-protein sulfhydryl compound in mammalian cells; it serves as a reservoir for cysteine and plays a vital role in protecting the cell from oxidative damage [7]. Peroxidation of multiple unsaturated fatty acids in biomembranes frequently occurs by exposure to reactive oxygen species. Malondialdehyde (MDA) arises from peroxidation of fatty acids, which have three or more double bonds. As one of the main by-products of lipid peroxidation, MDA is often used for assessment of oxidative damage. Determination of MDA levels provides a good measure of peroxidation, which is among the chief mechanisms of cell damage leading to necrosis [8]. In our study, we measured the levels of the endogenous antioxidant enzyme catalase (CAT), reduced glutathione (GSH), and MDA in pancreatic tissue samples by colorimetric kits for oxidative stress assessment.

Sample Preparations. After sacrification of the animals, pancreas was immediately removed, submerged into ice-cold normal saline, and homogenized in ice-cold Phosphate Buffer Saline (PBS) (0.01M, pH=7,4) using an ultrasonic homogenizer. The homogenate was used to assay levels of MDA, GSH, and to analyze CAT activity.

CAT activity assay.The CAT activities were measured by the method described by Beutler et al. This method is based on the rate of hydrogen peroxide decomposition by CAT contained in the examined samples. Absorbances were measured at 230 nm using a spectrometer. Tissue protein contents were measured using the Biuret method. The CAT activity was specified as units per milligram protein.

Determination of GSH level. Elabscience® GSH ELISA kit (Catalog No. E-EL-0026) was used to measure pancreatic GSH concentrations. The test was performed as per the protocol provided by the manufacturer. Briefly, pancreatic tissues were minced and homogenized in ice-cold PBS (0.01M, pH=7,4) and centrifuged at 5000 g at 4°C for 5 minutes (min). The supernatant was used to estimate the GSH concentration. Absorbance was read at 450 nm with a microplate reader. Tissue protein contents were measured by the Biuret method [2]. Levels were expressed as μg/mg protein.

Determination of MDA Level A. Elabscience® MDA ELISA kit (Catalog No. E-EL-0060) was used to analyze pancreatic tissue MDA concentrations. These analyses were performed as per the protocol provided by the manufacturer. Briefly, pancreatic tissues were minced and homogenized in ice-cold PBS (0.01M, pH=7,4) and centrifuged at 5000 g at 4°C for 5 min. The supernatant was used to estimate the GSH concentration. Absorbance was read at 450 nm with a micro-

plate reader. Tissue protein contents were measured by the Biuret method [2]. Levels were expressed as ng/mg protein.

Histopathological Evaluation. Formalin-fixed and dehydrated pancreatic tissues were embedded into the paraffin and sliced into sections (5-μm thickness), which were stained with hematoxylin and eosin by standard methods. Histopathological parameters including interstitial edema, intracellular vacuolization, neutrophil infiltration, congestion, hemorrhage and necrosis were analyzed for the assessment of the pancreatic injury. A scoring scale of 0 to 3 was used during these analyses: Negative (0), <30% (Score 1), 30-60% (Score 2), >60% (Score 3). At least 15 fields were examined for each section under x400 magnification, and the mean score was considered as the comprehensive evaluation for each animal.

Statistical Analysis. The data were statistically analyzed with IBM SPSS v23. The suitability of normal distribution was tested through Shapiro Wilk Test. One way analysis of variance and multiple comparison methods using Tukey HSD (Honestly Significant Difference) was implemented for comparisons between normally distributed matched data. Qualitative data were presented as mean±standard deviation and quantitative data as frequency (percent). The significance level was taken as $p < 0.05$.

Results. Mean serum BUN and Cr levels were significantly different among the groups ($p<0.001$) (Table 1).

Table 1

Comparison of parameters between the groups

	Bun	Creatine	Amylase	Lipase	MDA	CAT	GSH
Group 1	10.1 ± 4.7a	0.2 ± 0.02a	830.7 ± 482.3a	547.2 ± 68.6a	210.4 ± 52.8	7 ± 2.1	1393.3 ± 66.6
Group 2	31.2 ± 6.5c	0.7 ± 0.1b	1905.4 ± 310.7b	1664.5 ± 250.9b	289.3 ± 69.2	4.7 ± 0.9	1319.1 ± 119.5
Group 3	23.7 ± 2.3b	0.6 ± 0.1b	1462 ± 337.3b	1323.7 ± 325.2b	218.3 ± 62.5	6.8 ± 3.4	1318.3 ± 55.7
Group 4	29.9 ± 3.7bc	0.7 ± 0.1b	2002.7 ± 401.5b	1738 ± 372.3b	200 ± 33.3	6.6 ± 1.6	1277.8 ± 151.1
p	<0.001	<0.001	<0.001	<0.001	0.050	0.276	0.320

The arithmetic mean ± standard deviation, a-c: no difference between groups with the same letter

The lowest serum BUN level was detected in Group 1, and this level was statistically different from the other groups. There was also a significant difference between the mean BUN values of groups 2 and 3. This value was significantly lower in group 3 when compared with groups 2 and 4 ($p<0.001$) (see Table 1).

Serum creatinine value was lower in Group 1 than the other groups ($p<0.001$). There was no significant difference between groups 3 and 4 in terms of serum creatinine levels. Mean serum amylase levels are displayed in table 1. Statistical analysis revealed that mean serum amylase level was significantly lower in group 1 when compared with other groups ($p<0.001$) (see Table 1). On the other hand, there was no significant difference between groups 2, 3 and 4 in this regard. Serum lipase levels were significantly lower in Group 1 than in other groups ($p<0.001$).

Pancreatic tissue CAT, GSH and MDA levels are displayed in Table 1. Statistical analysis revealed that there was no significant difference between mean CAT, GSH and MDA values among the groups.

Frequency distributions of qualitative variables are presented in Table 2.

Table 2

The qualitative variables of the studied groups

	Group 1	Group 2	Group 3	Group 4	p
Interstitial edema					
Negative (Score 0)	4 (66.7)	1 (16.7)	3 (50)	1 (20)	0.011
Score 1	2 (33.3)	1 (16.7)	3 (50)	4 (80)	
Score 2	---	4 (66.7)	---	---	
Intracellular Vacuolization					
Negative (Score 0)	6 (100)	---	5 (93.3)	3 (60)	0.021
Score 1	---	2 (33.3)	1 (16.7)	2 (40.0)	
Score 2	---	2 (33.3)	---	---	
Score 3	---	2 (33.3)	---	---	

Continuation of Table 2

	Group 1	Group 2	Group 3	Group 4	p
Congestion and hemorrhage					
Negative (Score 0)	6 (100)	---	1 (16.7)	---	<0.001
Score 1	---	1 (16.7)	5 (83.3)	4 (80)	
Score 2	---	5 (83.3)	---	---	
Score 3	---	---	---	1 (20)	
Necrosis					
Negative (Score 0)	6 (100)	4 (66.7)	6 (100)	5 (100)	0.102
Score 1	---	2 (33.3)	---	---	

In terms of interstitial edema, 66.7% of the animals in group 1 had a negative (Score 0) result, and 33.3% had a score of 1. In group 2, 16.7% of the animals were negative (0), 16.7% and 66.7% had scores 1 and 2, respectively. In group 3, 50% was negative (0), while 50% had a score of 1. In group 4, 20% of the rats were negative (0) while 80% were scored 1.

All animals in group 1 were negative (0) in terms of intracellular vacuolization. In animals of Group 2, subjects with scores 1, 2 and 3 were equal in number (i.e., 33.3%). In group 3, 93.3% of the animals were negative (0), while 16.7% had a score of 1. In group 4, 60% were negative, while 40% had a score of 1.

Histopathological examinations revealed that 83.3% of the animals in Group 1 were negative, while 16.7% had a score of 1 in terms of intracellular vacuolization. In group 2, 16.7% were negative, while 50% had a score of 1. In this group, the rates of the animals which had scores 2 and 3 were both 16,7%. In group 3, 66.7% were negative, and the rates of scores 1 and 2 were both 16.7%. In group 4, 40% of the animals were negative, while 40% had a score of 1, and 20% had a score of 2.

All animals in group 1 were negative (0) in terms of congestion and hemorrhagia. Among the animals in group 2, 16.7% had a score of 1, while 83.3% had a score of 2. In group 3, 16,7% were negative, while 83,3% had a score of 1. In group 4, 80% of the animals had a score of 1, while 20% had a score of 3.

Histopathological examinations revealed that necrosis was negative (0) in all animals in groups 1, 2 and 4. In group 3, 33.3% of the animals in group 2 had a score of 1 (see Table 2).

Discussion. The main findings of our study indicate that renal I/R injury results in a significant elevation in serum BUN and creatinine, and it has remote effects on the pancreas, which express themselves with elevated serum amylase and lipase. However, there was no significant difference in concentrations of CAT, GSH and MDA in pancreatic tissues after renal I/R injury. To the best of our knowledge, this is the first study analyzing the effects of renal I/R injury on the pancreas and the potential preventive effects of different anesthetic agents in this setting.

Most of the research regarding renal I/R injury focused on the renal response to this injury. Hüssein et al. and Shun Yang et al. demonstrated that serum BUN and creatinine levels were significantly higher in animals that were subjected to renal I/R injury; 2h and 3 days after I/R, respectively [**1, 8**]. Yaoxian Liang et al. also demonstrated that serum BUN and creatinine levels significantly increased in the I/R group at 24h [**9**]. In line with the findings of Hüssein et al., our analysis revealed that serum BUN and creatinine levels were significantly higher in the ischemic group at 2h. In our study, serum BUN and creatinine levels significantly increased in the isoflurane preconditioning group compared to the sham group. This finding suggests that isoflurane preconditioning does not exert a protective role against I/R injury. Menting et al. reported that serum BUN and creatinine levels increased significantly in the ischemic group and isoflurane/IR group compared to the sham group [**10**]. In this study, serum BUN levels were lower in ISO/IR group, but the difference was not significant. Our results are in accordance with the findings of Menting et al. in terms of serum Cr levels. In our study, we found a significant difference between the sham group and epidural bupivacaine group in terms of serum BUN and creatinine levels.

There are only a few studies reported in the literature regarding the effects of renal I/R injury on the pancreas. In an experimental study, Hüssein et al. reported that serum amylase levels significantly increased 2 hours, 1 day and 3 days after renal I/R injury [1]. Also, they detected that serum lipase levels significantly increased on days 1 and 3. In accordance with Hüssein et al., we identified that renal I/R injury led to a significant increase in serum amylase and lipase levels. Our results revealed that serum amylase and lipase levels were higher in the animals, which were anesthetized by bupivacaine than in the animals which were anesthetized by isoflurane; however, the difference between the two groups was not statistically significant. Hüssein et al. reported that pancreatic tissue MDA levels were high only 2 hours after ischemia-reperfusion, while pancreatic tissue CAT levels were high at all time intervals in the ischemic group and GSH levels were low only 2 hours after I/R [**1**]. In our study, we found that MDA levels were higher in the renal

I/R injury group than the other groups, but the difference was not significant. In the study of Hüssein et al. pancreatic edema, leukocyte infiltration and vacuolization were significantly increased in the ischemic group. While pancreatic necrosis was observed 2 hours after renal I/R in the ischemic group, pancreatic hemorrhage was not detected in any group [1]. We found that the severity of pancreatic tissue interstitial edema, intracellular vacuolization, congestion and hemorrhage were significantly higher in the renal I/R group than in the sham group, but there was no difference in neutrophil infiltration and necrosis. Although there are several studies about the protective effect of volatile anesthetics and epidural anesthesia against renal I/R injury, there is no study in the literature that investigated the potential protective effects of these anesthetic agents on the pancreas in the setting of renal I/R injury.

Carraretto et al. reported that propofol and isoflurane had protective effects against renal I/R injury [2]. Lee et al. observed that serum creatinine level was significantly lower in animals anesthetized with sevoflurane, isoflurane and halothane compared to the group anesthetized with phenobarbital and ketamine [3]. They concluded that volatile anesthetics were protective against renal I/R injury. Liang et al. stated that isoflurane was protective against I/R injury of the heart, liver and brain, and they demonstrated that isoflurane improved the renal I/R injury by inhibiting mitochondrial apoptosis [9]. Menting et al. reported that repetitive administration of isoflurane daily, exerted a protective effect against renal I/R injury while single-dose isoflurane did not have the same effect [10]. Acar et al. compared the protective effects of spinal and epidural anesthesia against I/R injury created in transverse rectus abdominis muscle and reported that epidural anesthesia is superior to spinal anesthesia in preventing I/R injury [11].

There are reports suggesting that local anesthetic infusion does not prevent and even increases renal I/R injury [12]. Sarikus et al. investigated the effects of epidural bupivacaine administration on hepatic I/R injury. They reported that epidural bupivacaine administration did not prevent inflammatory response and lipid peroxidation in rat livers in the setting of hepatic I/R injury [13]. Lee et al. proposed that continuous local anesthetic infusion worsened the renal damage in rats with renal I/R injury, and renal cell death and inflammatory changes were significantly more prominent in rats treated with local anesthesia [14]. These authors showed that local anesthetic infusion enhanced renal I/R injury by increasing necrosis, apoptosis, and inflammation.

We found that serum BUN levels of the epidural bupivacaine-administered group were significantly lower than BUN levels of the renal I/R group. However, there was no significant difference between bupivacaine and isoflurane administered groups (Groups 3 and 4) and renal I/R group (Group 2) in terms of serum creatinine, amylase, lipase and pancreatic tissue MDA, CAT and GSH levels as well as histopathological parameters.

Conclusions. In conclusion, we detected that renal function is impaired, morphology and exocrine functions of the pancreas are adversely affected 45 minutes after bilateral renal I/R. However, this impact was not significant. Moreover, the administration of epidural bupivacaine during ischemia-reperfusion had a positive effect only in terms of serum BUN levels, and it did not have any influence on other parameters. On the other hand, the effects of anesthesia with isoflurane and ketamine were similar to each other. We believe that these findings should be supported with experimental studies performed on larger samples.

Conflict of Interest Statement. The authors declare no conflict of interest in this study.

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Authors contribution. *ZAE*, and *TU* contributed to the design and implementation of the research, analysis of the results and writing of the manuscript.

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