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Silymarin Ameliorates Valproic Acid-Induced Pancreas Injury by Decreasing Oxidative Stress

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Abstract
In this study, the protective effects of silymarin (SLY) against valproic acid (VPA) induced pancreatic damage was investigated in rats by using histological and biochemical methods. The experiment was performed with 21 Sprague Dawley male rats. Rats were divided into three groups: The control group received 0.5 mL saline via gavage, VPA group received 500 mg/kg/day VPA by gavage and VPA+SLY group received 500 mg/kg/day VPA+ 100 mg/kg/day SLY via gavage for 14 days. Serum amylase and lipase levels, which increased as a result of VPA application, were reversed with SLY application. In addition, with VPA administration, malondialdehyde (MDA) increased and glutathione (GSH) levels decreased in pancreatic tissue. This situation was significantly ($p \leq 0.05$) suppressed with the application of SLY. Histopathological changes such as acinar cell degeneration, bleeding, infiltration, necrosis and shrinkage in Langerhans islets were observed after VPA application. Reactive oxygen species (ROS) and histopathological changes decreased and antioxidant activity increased in VPA + SLY group compared to VPA group. The results obtained in this study revealed that SLY is a potential pharmacological agent that can be used as a protective and therapeutic agent against VPA-induced pancreatic damage.

Keywords: Pancreas injury, rat, silymarin, valproic acid.

INTRODUCTION

The chemical structure of VPA contains an eight-carbon fatty acid called propyl acetate. The mode of action is in the synaptic area; gamma-aminobutyric acid (GABA) inhibits transaminases that break down, reduce GABA reuptake and act by increasing the amount of GABA in this area (Holland, 2001). Because VPA epilepsy affects many types of seizures, it is widely used in both partial and generalized epilepsies (Panayiotopoulos, 2005). It is also used for the treatment of diseases such as brain neoplasm, migraine, bipolar, mania and psychiatric disorders (Ahangar et al., 2017; Semmler et al., 2017). Long-term VPA use, especially in children, has been reported to cause acute pancreatitis (Pellock et al., 2002). The mechanism of VPA administration to create acute pancreatitis has not been fully elucidated. However many researchers claim that there are ROS that increase due to the depletion of antioxidant enzymes. The increased ROS level cause the pancreatitis via the toxic effect on the cell membranes of the pancreas (Sanfey et al., 1984; Pippenger et al., 1991; Oktay et al., 2017). Eisses et al. (2015) claimed that VPA does not directly cause pancreatitis. They claimed that VPA inhibits histone deacetylase (HDAC), which controls acinar cell proliferation, which plays a role in the pancreatic regeneration, causing delays in pancreatic healing.

SLY is an active ingredient in the milk of thistle (*Silybum marianum*), obtained by standard extraction of the plant seeds (Sokar et al., 2017). SLY has antioxidant, anti-inflammatory, protein synthesizer and anti-fibrotic properties. It also shows protective effects on liver, pancreas, kidney, myocardium and central nervous system (Soto et al., 2004). The pharmacological agents used in the studies caused some histopathological changes e.g. acinar cell degeneration, hemorrhage, infiltration, necrosis and shrinkage in Langerhans islets in the pancreas. In these histopathological changes, healing was observed due to the protective feature of SLY (Kim et al., 2020). In addition, SLY has a ROS scavenger and membrane stabilizer feature. It inhibits autophagy caused by ROS in the pancreas and repairs damaged cells (Soto et al., 1998; Ying et al., 2019). The mechanism of this antioxidant effect is estimated to prevent the formation of diabetes by directly cytoprotective effect on critical INS-1 cells that prevent apoptosis in β cell in the pancreas (Yang et al., 2018).

In this study, the protective effects of SLY against VPA induced pancreatic damage were investigated using histological and biochemical parameters in rats. No studies about that the effect of SLY against VPA-induced pancreatitis could have been found in the literature. In this study, possible therapeutic and protective effects of SLY against VPA-induced pancreatic damage in rats were investigated.

MATERIALS AND METHODS

Chemicals

VPA and SLY were purchased from Liba Co (Turkey) and Madaus (Turkey), respectively. Hydrochloric acid (HCl), trichloroacetic acid (TCA), thiobarbituric acid (TBA) and paraffin were obtained from, Sigma-Aldrich (USA). Xylene, hematoxylin-eosin and ethanol were obtained from Merck (Germany). All other chemicals which had the analytical grade were purchased from Sigma-Aldrich (USA).

Animals

This study was carried out in Adıyaman University Experimental Animal Production Application and Research Center. Provided from this center; 21 male Sprague-Dawley rats (220-240 g, 8 weeks old) were utilized. Ethics committee report was taken from the Firat University

Faculty of Medicine Laboratory Animal Ethics Committee (Protocol 2019/10). The study was performed according to this protocol.

Treatment protocol

In the experiment, 21 rats were used. Seven rats were placed randomly in each group. It was divided into three groups. The control group received 0.5 mL saline via gavage, VPA group received 500 mg/kg/day VPA by gavage and VPA+SLY group received 500 mg/kg/day VPA+ 100 mg/kg/day SLY via gavage for 14 days (Tong et al., 2005; Beydilli et al., 2015). At the end of the 14th day, after the rats were taken under anesthesia with (75 mg/kg + xylazine 10 mg/kg i.p). They were sacrified by cervical dislocation. Blood samples were collected from the jugular vein. Serums were obtained by centrifuging at 5,000 x g for 15 minutes. Stored at -86 °C for biochemical analysis. The pancreas was excised. For histopathological analysis, it was fixed in 10% buffered neutral formalin solution for 24 hours at +4 °C.

Biochemical evaluation

Serum biochemical parameters (amylase mg/dL and lipase mg/dL) were analyzed on the Olympus 2700 analyzer (Olympus Diagnostica GmbH, Hamburg, Germany). The analysis was conducted according to the Reitman-Frankel colorimetric transaminase method (Crowley, 1967).

Oxidative stress biomarkers

MDA level was analysed in pancreatic tissue. The sample and stock solution (0.375% thiobarbituric acid in 0.25 N HCl and 15% trichloroacetic acid) were mixed (1:1, v/v) in a centrifuge tube. The solution was heated for 15 minutes in boiling water and then cooled. The residue was centrifuged at 2,500 x g for 10 minutes. Samples were measured with a spectrophotometer at the wavelength of 532 nm (Placer et al., 1966).

GSH levels in pancreatic tissues were determined according to Sedlak and Lindsay (1968) method. The sample was eluted with 50% TCA and centrifuged at 1,000 x g for 5 minutes. 2 mL Tris-EDTA buffer (0.2 M, PH = 8.9) and 0.1 mL 0.01 M 5,5'-dithio-bis-2 by taking 0.5 mL of the supernatant from the supernatant-nitrobenzoic acid was added. The mixture of sample was kept for 5 minutes at room temperature. It was measured with the spectrophotometer at the wavelength of 412 nm.

Histopathological examinations

Pancreatic tissues were fixed in a 10% neutral formalin solution. Tissues were washed overnight in running water. The washed samples were dehydrated with ethanol, cleaned with xylene and embedded in paraffin. 4-5 µm thick sections were taken from the paraffin blocks (RM2125RTS, Leica, Germany). Sections of 4-5 µm thickness were taken from the waxed blocks (RM2125RTS, Leica, Germany). Samples were stained with hematoxylin and eosin for histopathological evaluation. Histopathological scoring was performed in terms of acinus degeneration with cell infiltration, degenerative shape of islets (shrinkage), vascular congestion and hemorrhagic areas by examining different areas in 40x objective from each section. According to the grade of the findings (-): no findings, (+): low level, (++): moderate level, (+++): severe level. Samples were viewed in a binocular light microscope (ECLIPSE Ni-U, Nikon, Tokyo, Japan). These images were evaluated according to Refaiy et al. (2011) method.

Statistical analyze

SPSS 20.0 version was used in the statistical analysis of this study. Shapiro-Wilk test was used in normality evaluation. One-way ANOVA, post-hoc and LSD were applied for intra-group comparisons of parametric data. Kruskal Wallis test was used in biochemical data. The same test was used for the semi-qualified evaluation of histopathological scores and for differences in data measured between groups. Mann-Whitney U test was used to compare the data of the three groups. Data were considered statistically significant for $p \leq 0.05$.

RESULTS

Biochemical evaluation

Amylase and lipase levels increased significantly in the VPA group compared to the control ($p \leq 0.02$) and VPA + SLY groups ($p \leq 0.04$). SLY administration caused a significant decrease ($p \leq 0.05$) in VPA-induced amylase and lipase levels (Table 1).

Table 1. Serum biochemical and pancreas tissue oxidative stress biomarkers of the experimental groups.

	CONTROL	VPA	VPA + SLY	P VALUES
Serum biochemical biomarkers				
Amylase ml/dL	1043.14 ± 6.57	2094.57 ± 18.52[a]	1228.57 ± 38.35[b]	p <0.002
Lipase ml/dL	12.71 ± 0.83	20.71 ± 0.86[a]	14.71 ± 0.68[b]	p <0.004
Tissue oxidative stress biomarkers				
GSH (µmole/gr tissue)	440.00 ± 3.27	379.71 ± 2.44[a]	428.33 ± 4.55[b]	p <0.003
MDA (nmole/gr tissue)	229.71 ± 2.42	301.00 ± 1.63[a]	240.00 ± 1.29	p <0.005

Data are means ± SEM, n = 7. [a] VPA increased pancreatic damage vs. control. [a] $p < 0.04$, [b] SLY reduced pancreatic damage vs. VPA [b] $p < 0.05$. VPA, valproic acid; SLY, silymarin; GSH, glutathione; MDA, malondialdehyde.

Oxidative stress biomarkers

Significantly higher MDA levels ($p \leq 0.03$) and significantly lower GSH levels ($p \leq 0.02$) were detected in the VPA group compared with the control group. With SLY application, a significant decrease in MDA level ($p \leq 0.04$) and a significant increase in GSH level ($p \leq 0.03$) were observed (Table 1).

Histopathological results

The structural changes in the pancreatic tissue sections of the control and experimental groups were scored (Figure 1, Table 2). Histological examination of the pancreatic tissue sections of the control group (group I) revealed no findings other than normal histological structures (Figure 1 A-B, Table 2). VPA group (group II), when the pancreatic tissue is examined according to the control group; acinus degeneration with cell infiltration, degenerative shape of islets (shrinkage), vascular congestion, hemorrhagic areas were observed (Figure 1 C-D, Table 2). VPA and SLY group (group III) showed an improvement in histopathological findings compared to the VPA group (group II) (Figure 1 E-F, Table 2).

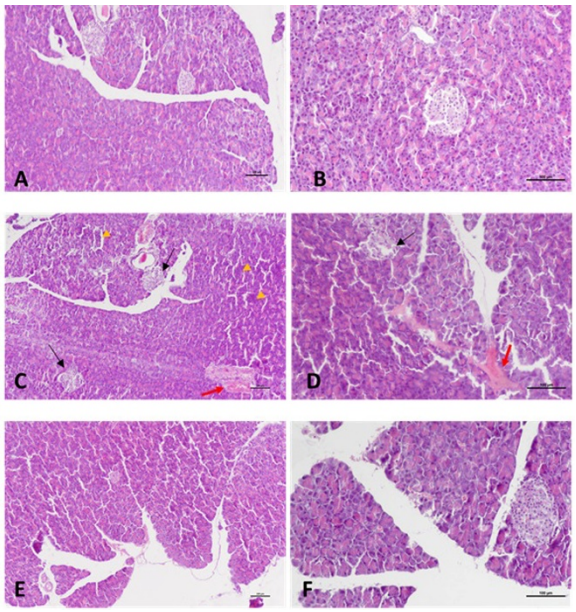

Figure 1. Pancreatic tissue sections of control and experimental groups.**A-B:** The control group (group I) showed normal histological appearance in the pancreatic tissue sections. **C-D:** In the pancreatic tissue sections of the VPA group (group II); shrinkage islets of langerhans (black arrows), degenerative acinuses and surrounding mononuclear cell infiltrations (yellow arrowheads) and hemorrhagic areas - vascular congestions (red arrows) were observed. **E-F:** Pancreatic tissue sections of the VPA + SLY (group III); A decrease in histopathological findings was observed compared to Group II (H–E, A – C - E x10, B – D - F x20).

Table 2. Histopathological scoring of pancreas sections of experimental groups.

Parameters/scores	CONTROL	VPA	VPA + SLY
Acinus degeneration with cell infiltration	–	$+++^{a}$	$++^{b}$
Degenerative shape of islets (shrinkage)	–	$+++^{a}$	$+^{b}$
Vascular congestion	–	$++^{a}$	$+^{b}$
Hemorrhagic areas	–	$++^{a}$	$++^{b}$

DISCUSSION AND CONCLUSION

Long-term use of VPA for the treatment of epilepsy has been reported to cause many side effects in the liver, kidneys, pancreas, central nervous system and hemopoietic system (Belcastro et al., 2013). In the literature, the effectiveness of many antioxidant agents has been investigated against the negative effects of VPA on pancreas, but the protective efficacy of SLY has not been studied (El-Deeb, 2006). Therefore, in the present study, possible therapeutic and protective effects of antioxidant SLY against VPA-induced pancreas toxicity were investigated using biochemical and histopathological methods.

Amylase; a small amount of salivary glands, the vast majority is a digestive enzyme synthesized by the pancreas. This protein enzyme makes carbohydrates ready for absorption by breaking down in the small intestine. Lipase is responsible for the breakdown of fats with nutrients up to building blocks and making them usable in the body. Thanks to this enzyme, the fats that progress in the digestive tract by being taken into the body in the form of triglycerides are converted into fatty acids and glycerol and are ready for absorption. (Özcan, 2020). Serum amylase and lipase levels increase in acute pancreatitis (Sevinç, 2006). In the present study, serum amylase and lipase levels were found high. Gerstner et al. (2007) observed that amylase and lipase levels increased in patients with pancreatitis as a result of VPA administration. These increased serum amylase and lipase levels were reversed by SLY administration. Kim et al. (2020) found that the high amylase level reduced with SLY administration in acute pancreatitis caused by cerulein.

Oxidative stress is the shift of balance between ROS and antioxidants in the body to the ROS side. MDA is a product of lipid peroxidation and an important indicator of ROS (Shoji and Koletzko, 2007). The level of ROS in the body is regulated by enzymatic and non-enzymatic antioxidant systems. GSH is a non-enzymatic antioxidant. ROS plays an important role in the pathogenesis of DNA (Arora and Singh, 2014; Osborne and Stanton, 2005; Xu et al., 2005; Roshan and Stanton, 2013). In previous studies, it was reported that after the use of VPA, the amount of MDA level increased and the GSH level reduced in the pancreatic tissue (Underwood and Freye, 1993). In our study, it is clearly seen that VPA decreases GSH level and increases MDA level in the pancreatic tissue. Our findings show that VPA causes oxidative stress in the pancreas and is compatible with the literature on this subject. We also noted that with SLY application, GSH and MDA levels reached a value close to those in the control group. This result showed that the use of SLY greatly reduced the ROS shaped in pancreatic tissue after VPA administration. In studies conducted, they reported that SLY decreased ROS and increased GSH levels (Valenzuela et al., 1989; Soto et al., 2003). The data obtained in the present study is in parallel with the literature on this subject and confirms the antioxidant effects of SLY.

In previous studies, it has been reported that long-term use of VPA for treatment caused acute pancreatitis in children (13%), and the risk of acute pancreatitis increased proportionally with dosage (Pellock et al., 2002). In addition, in experimental studies with mice and rats, VPA administration has been reported to cause chronic atrophic pancreatitis, decrease acinar cell proliferation, delay the healing of damaged pancreas, and cause degenerative changes in Langerhans islets and acinar cells (Walker et al., 1990). In the present study, histopathological changes such as acinus degeneration with cell infiltration, degenerative shape of islets (shrinkage), vascular congestion, hemorrhagic areas were observed in the pancreas of VPA group rats. These changes are compatible with the literature mentioned above and confirm the pathological effects of VPA on pancreas. It is clearly observed in our study that histopathological changes caused by VPA are improved by SLY. Therefore, this result can be seen as a proof of the protective effect of SLY on the pancreas. SLY with antioxidant, anti-inflammatory, antiapoptotic, anticarcinogenic, antiviral, antifibrotic and antiangiogenic effects especially used in the treatment of liver diseases (Ramakrishnan et al., 2009; Ying et al., 2013). Kim et al. (2020) generated acute pancreatitis in an experimental study with celurein.They determined histopathological changes such as edema, inflammatory cell infiltration, necrosis and vacuolation in acinar cells, neutrophil infiltrationin pancreas tissue. Recent studies in this area reported that SLY application greatly improved these shaped pathological changes. Soto et al. (2004) conducted another experimental study by applying alloxan. They reported pathological changes such as islets shrinkage, ß-cell loss, necrotic areas, lipid accumulation, hemorrhage, and lymphocyte infiltration were observed in pancreas tissue. It was also stated in this study that almost pathological changes completely recovered after SLY administration. The protective effects of SLY on pancreas tissue match the findings obtained in the present study. The mechanism of formation of VPA induced acute pancreatitis

has not been fully elucidated. However, it has been claimed that the ROS that increase as a result of the depletion of antioxidant enzymes are shaped by the direct toxic effect of the pancreas on the cell membranes (Perucca and Gilliam, 2012). According to the present study, the current findings in the VPA group; the biochemical and histopathological results obtained regarding the increase of ROS and tissue damage in pancreas tissue support this claim.

As a result, the data obtained in the present study revealed that SLY improved VPA-induced damage in pancreas tissue in rats. SLY is able to achieve these effects, possibly by preventing lipid peroxidation, by increasing antioxidant enzymes or by other mechanisms. The results obtained in the present study were revealed that SLY is a therapeutic pharmacological agent to prevent pancreatic damage. However, more studies are needed to reveal the mechanisms of action underlying the protective and therapeutic effects of SLY.

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Conflict of Interest
The authors declare that there is no conflict of interest in the content of the article

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