The effects of a Cilostazol, a selective phosphodiesterase III inhibitor, on liver ischemic-reperfusion injury and liver regeneration; In vitro experimental study

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ABSTRACT

Objective: Hepatectomy and transplantation cause liver damage through ischemic reperfusion and oxidative stress. There is no treatment available to improve liver regeneration and reduce ischemic-reperfusion injury. The present study aimed to investigate whether a selective phosphodiesterase III inhibitor, Cilostazol, improves ischemic reperfusion injury and liver regeneration following extended hepatectomy.

Material and Methods: Wistar albino rats (n=40) were randomized and divided into 4 equal groups. All rats underwent 60% hepatectomy, and Cilostazol (5 mg/kg per day) was administered to the experimental group. The subjects were sacrificed on the 4th and 7th days following the resection. Blood samples were taken to evaluate liver enzymes (ALT, AST) and liver tissue samples were taken to analyze morphology. Biochemical, morphological, and histopathological parameters were compared between Groups.

Results: No statistically significant differences were detected in ALT, AST values, and relative liver weights in rats treated with Cilostazol compared to the control group without Cilostazol. Although not statistically significant, a significant increase was detected in relative liver weight and a decrease in AST value in rats treated with Cilostazol. SOD activity was found to be significantly higher and GSH levels, MPO and AOPPs levels were significantly lower in Cilostazol applied Groups. It is seen in these findings that selective inhibition of PDE3 by Cilostazol improves hepatic circulation. It was also found that ischemic reperfusion injury decreased and regeneration markers such as mitosis index, even nucleus, and proliferating cell nuclear antigen ratio increased in rats treated with Cilostazol.

Conclusion: The present study found that selective PDE3 inhibitor Cilostazol positively affected the histopathological parameters following extended liver resection and significantly increased hepatocellular proliferation.

Keywords: Cilostazol, ischemic-reperfusion injury, liver regeneration

INTRODUCTION

Liver damage can occur due to many different mechanisms through hepatectomy, transplantation, hypovolemia, cardiogenic shock cause liver damage, Ischemic-Reperfusion Injury (IRI), and oxidative stress (1). Both major hepatectomy and liver transplantation result in significant hemodynamic changes. The inhibition of apoptosis, acute inflammatory process, oxidant damage, and providing oxygenation following hepatectomy and liver transplantation seem to be effective methods to protect the liver against IRI (2-3). Animal model studies showed that drip infusion of phosphodiesterase-3 (PDE3) inhibitors reduces hepatic IRI (4).

Cilostazol, which is a selective PDE3 inhibitor, is a type III phosphodiesterase (PDE3) inhibitor with partial type V phosphodiesterase activity and was administered to increase the level of cyclic adenosine monophosphate (cAMP) (5). PDE3 inhibitors have anti-platelet aggregation effects by inhibiting platelet function (6). It is also known that PDE3 inhibitors increase Nitric Oxide (NO) release from endothelial cells and cause vasodilatation (7). It has also been reported that PDE3 inhibitors offer benefits in hepatic IRI through mechanisms such as increased blood flow to hepatic tissues, platelet anti-aggregation effects, and anti-inflammatory effects (8).
The objective of the current study was to explore the pharmacological effects of the selective PDE3 inhibitor Cilostazol on hepatic ischemic-reperfusion injury and liver regeneration in an animal model of major hepatic resection.

MATERIAL and METHODS

The study had an experimental animal design and was conducted in a laboratory environment. In future surgical procedures, examining the effect of Cilostazol on liver regeneration for the continuation of hepatic functions following heptectomy may provide new and improved possibilities in treatment. The present study was conducted in Zonguldak Bülent Ecevit University Medical Faculty Medical and Surgical Research Center with the permission of the Experimental Research Ethics Committee with the number B.30.2.Z.K.Ü.0.00.00.00/33.

Forty Wistar Albino rats, aged 20 weeks and weighing between 200 to 270 grams, were employed as the experimental subjects. In all groups, 60% of the liver was surgically resected. The study comprised a total of four groups, each consisting of ten rats. Groups 1 and 2 were designated as control groups, while Groups 3 and 4 were categorized as experimental groups. Table 1 presents the detailed characteristics of each group.

One rat died in each group during the experiment. Anesthesia was provided with 90mg/kg ketamine in rats. Following cleaning, the abdomen was entered with a median incision. The standard heptectomy technique of Higgins and Anderson was employed for this purpose in which 60% of the liver is resected (9). The Left and median lobes of the liver were resected. The right liver lobe and caudate lobe were left in all rats. Cilostazol was administered to the experimental groups at a daily dose of 5 mg/kg. According to previous research, mitosis initiation takes place within 24-30 hours subsequent to a 60% heptectomy in rats, with the peak mitotic rate occurring between the 4th and 7th days. In light of these findings, the current study employed sacrificial procedures on both the 4th and 7th days to assess liver regeneration.

Sacrification was performed with the intracardiac blood collection method. The liver tissue was excised completely and preserved in 10% formaldehyde for histopathological examinations and tissue enzyme determinations. Aspartate Aminotransferase (AST, U/L) and Alanine Aminotransferase (ALT, U/L) were studied from sera obtained from the rats. BioSystems Reagents and Instruments kits (BioSystems S.A., Barcelona, Spain) were used. Myeloperoxidase (MPO, U/gr), and superoxide dismutase (SOD, U/mg) activity were measured from tissue enzyme levels (10-11). Advanced Oxidation Protein Products (AOPPs, μmol/L), protein determination (mg/mL), and total sulphydryl content were calculated over the GSH curve (μmol/mg) (12-13-14).

From morphological parameters, Relative Liver Weight Measurement (RLW) was calculated as liver weight at autopsy - (whole liver weight - resected liver weight) x 100. Mitosis Index (MI) from histopathological parameters, and the rate of labelling with Proliferating Cell Nuclear Antigen (PCNA) (15).

Statistics

The SPSS 22.0 statistical software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. Descriptive statistics mean ± Standard Deviation (SD) descriptive analysis measures were used for patient characteristics and variables. The Mann-Whitney U test was employed for group comparisons, given that parametric test assumptions were not met, and a significance level of p<0.05 was deemed statistically significant.

RESULTS

The present study was conducted on Wistar Albino rats (n=40). Groups 1 and 2 were the control group in the study. The rats in these groups were not given Cilostazol. Groups 3 and 4 were the experimental groups. The rats in these groups were administered daily Cilostazol at 5 mg/kg. The experimental and control groups (Groups sacrificed on day 4 and Groups sacrificed on day 7) were compared, and the experimental group (Group 3, Group 4) and the control groups (Group 1, Group 2) were also compared.

No statistically significant differences were observed in the levels of the liver enzymes ALT and AST when comparing the experimental and control groups. However, the AST value was lower in the experimental groups. When the experimental and control groups were compared, ALT values were insignificant. Regarding AST, when the control groups were compared, the AST value was lower in Group 2 and was found to be statistically significant. The findings are given in Tables 2 and 3.

When comparing the experimental and control groups with respect to SOD activity, Group 3 exhibited a statistically significant increase in SOD activity. Among the control groups, Group 2 showed a significant elevation in SOD activity. In contrast, within the experimental groups, Group 4 displayed a significant rise in SOD activity.

Regarding MPO levels, a significant decrease in MPO was observed in Group 4 when comparing the experimental and control groups. Conversely, within the experimental groups, Group 3 demonstrated a significantly higher MPO value.

In terms of AOPPs, both Group 3 and Group 4 exhibited statistically significant reductions in AOPPs values compared to the control group when analyzing the experimental and control groups. Similarly, within the experimental groups, Group 4 displayed a significantly lower AOPPs value.

Analyzing GSH values, a noteworthy reduction was observed in Group 4 when comparing the experimental and control groups. This reduction in GSH value was also significant when analyzing the experimental groups alone.

Other comparisons yielded insignificant findings. Detailed results can be found in Tables 2 and 3.

No significant differences were detected between all groups in terms of the relative weight of the liver. In terms of histopathological mitosis index, the experimental groups (Groups 3 and 4 treated with Cilostazol) had a statistically significantly higher Mitotic Index than the control groups (Groups not treated with Cilostazol; Groups 1 and 2) (Figure 1 1).

Medical Science and Discovery, 2023; 10(8):546-551
When the experimental groups were compared, a more statistically significant Mitosis Index was found in Group 4 and Group 2 than in the control Groups. When the experimental and control groups were compared, double nuclei were found to be significantly more common in Group 4 (Figure 2). Cilostazol increased the Mitosis Index and the number of double nuclei.

The PCNA ratio was significantly higher in the experimental groups (Group 3, Group 4) (Figure 3). When the experimental groups were compared, a higher PCNA rate was found in Group 4 and Group 2 compared to the control groups. Other findings in the comparisons were insignificant. The findings are given in Tables 2 and 3.

Table 1: Characteristics of the Groups

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group 1 n=10</th>
<th>Group 2 n=10</th>
<th>Group 3 n=10</th>
<th>Group 4 n=10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepatectomy, 60% resection of the liver</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Cilostazol, 5 mg/kg/day</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sacrification, day</td>
<td>4th day</td>
<td>7th day</td>
<td>4th day</td>
<td>7th day</td>
</tr>
</tbody>
</table>

Table 2: ALT, AST, tissue enzymes (MPO, SOD), oxidation protein products, total sulfhydryl content (AOPPs, GSH), and histopathological data of the Groups that did not receive Cilostazol (Groups 1 and 2) and Groups that received Cilostazol (Groups 2 and 4).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group 1 n=10</th>
<th>Group 2 n=10</th>
<th>Group 3 n=10</th>
<th>Group 4 n=10</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT, U/L</td>
<td>46.5±6.8</td>
<td>43.8±5.6</td>
<td>63.4±6.6</td>
<td>59.7±7.8</td>
</tr>
<tr>
<td>AST, U/L</td>
<td>174.4±12.4</td>
<td>158.6±10.4</td>
<td>167.5±13.2</td>
<td>151.7±11.1</td>
</tr>
<tr>
<td>MPO, U/g</td>
<td>0.20</td>
<td>0.22</td>
<td>0.21</td>
<td>0.13</td>
</tr>
<tr>
<td>SOD, U/mg</td>
<td>113.1±24.5</td>
<td>129.1±16.9</td>
<td>146.1±18.5</td>
<td>107.9±23.3</td>
</tr>
<tr>
<td>AOPPs, μmol/L</td>
<td>364.7±35.7</td>
<td>603.7±42.7</td>
<td>248.2±36.2</td>
<td>164.0±25.7</td>
</tr>
<tr>
<td>GSH, μmol/mg</td>
<td>298.7±26.3</td>
<td>328.1±31.5</td>
<td>343.5±28.3</td>
<td>237.9±28.2</td>
</tr>
<tr>
<td>RKA</td>
<td>36.3±13.4</td>
<td>44.1±12.5</td>
<td>41.4±13.3</td>
<td>51.9±12.9</td>
</tr>
<tr>
<td>ML, %</td>
<td>3.5</td>
<td>12.6</td>
<td>5.4</td>
<td>19.3</td>
</tr>
<tr>
<td>Even nucleus, %</td>
<td>15.4</td>
<td>18.6</td>
<td>17.8</td>
<td>27.3</td>
</tr>
<tr>
<td>PCNA, %</td>
<td>15.2</td>
<td>26.6</td>
<td>21.9</td>
<td>39.3</td>
</tr>
</tbody>
</table>

Table 3. Statistical comparison of ALT, AST, tissue enzymes (MPO, SOD), oxidation protein products, total sulfhydryl content (AOPPs, GSH), histopathological data of the groups that did not receive Cilostazol (Groups 1 and 2), and groups that were administered Cilostazol (Groups 2 and 4) results

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group 1 and Group 3 (p value)</th>
<th>Group 2 and Group 4 (p value)</th>
<th>Group 1 and Group 2 (p value)</th>
<th>Group 3 and Group 4 (p value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALT, U/L</td>
<td>0.113</td>
<td>0.114</td>
<td>0.161</td>
<td>0.489</td>
</tr>
<tr>
<td>AST, U/L</td>
<td>0.796</td>
<td>0.120</td>
<td>0.019</td>
<td>0.863</td>
</tr>
<tr>
<td>MPO, U/g</td>
<td>0.863</td>
<td>0.001</td>
<td>0.666</td>
<td>0.003</td>
</tr>
<tr>
<td>SOD, U/mg</td>
<td>0.001</td>
<td>0.136</td>
<td>0.002</td>
<td>0.024</td>
</tr>
<tr>
<td>AOPPs, μmol/L</td>
<td>0.040</td>
<td>0.001</td>
<td>0.666</td>
<td>0.011</td>
</tr>
<tr>
<td>GSH, μmol/mg</td>
<td>0.161</td>
<td>0.063</td>
<td>0.605</td>
<td>0.031</td>
</tr>
<tr>
<td>RKA</td>
<td>0.297</td>
<td>0.161</td>
<td>0.113</td>
<td>0.436</td>
</tr>
<tr>
<td>ML, %</td>
<td>0.043</td>
<td>0.004</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>Even nucleus, %</td>
<td>0.297</td>
<td>0.011</td>
<td>0.094</td>
<td>0.001</td>
</tr>
<tr>
<td>PCNA, %</td>
<td>0.006</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
</tr>
</tbody>
</table>

p<0.05 was taken as the limit of significance.

Figure 1. Histopathological staining with Hematoxylin-Eosin (200x), mitosis index (a) in Group 1 from the Groups that did not receive Cilostazol, and mitosis index in Group 3 from the Groups that received Cilostazol (b).
DISCUSSION

It is well established that the liver has an extraordinary capacity to regenerate and regain its former functional abilities after extensive resections [16]. The extent of the regenerative process depends on the size and the amount of resected tissue, and therefore, an experimental 60% partial hepatectomy model reveals a well-regulated liver cell proliferation process modulated by a wide variety of cytokines and growth factors (17). It was shown that the remaining liver tissue regeneration starts from the first day following partial hepatectomy. Significant regeneration occurs in the first 10 days. The present study was planned in line with this clinical information.

The present study found positive effects on ischemic reperfusion and increased rates of regeneration markers such as mitosis index, double nucleus, and proliferating cell nuclear antigen in the Groups administered Cilostazol. The study showed that the selective phosphodiesterase III inhibitor Cilostazol can increase liver regeneration following 60% liver resection.

No statistically significant differences were detected in relative liver weights and ALT and AST values in rats treated with Cilostazol compared to the control group without Cilostazol. In rats administered Cilostazol, there was a significant increase in relative liver weight, although not statistically significant. In a previous study, AST level was shown to be a good clinical indicator of ischemic-reperfusion injury (18-19). In the present study, AST was lower in rats treated with Cilostazol.

Considering the pharmacological effects of PDE3 inhibitors, they can affect the ischemic-reperfusion injury and liver regeneration process. A previous study showed that the PDE3 inhibitor amrinone improved liver regeneration in a rat model with 70% liver resection. The authors hypothesized this may be because of improved hepatic blood flow and sinusoidal perfusion (20). They also found increased mitosis index, double nucleus, and proliferating cell nuclear antigen ratio, similar to the present study in which it was found that hepatocellular proliferation caused the most significant increase on the 7th day following hepatectomy.

Figure 2: Histopathological staining with Hematoxylin-Eosin (200x), even nucleus (a) in Group 2 from the Groups that did not receive Cilostazol and even nucleus (b) in Group 4 from the Groups that received Cilostazol.

Figure 3: PCNA ratio (a) in Group 1 from the Groups that did not undergo Cilostazol in immunohistochemical staining with the PCNA (Proliferating Cell Nuclear Antigen) (Thermo Scientific, Fremont, CA, USA) kit and the PCNA ratio in Group 3 from the Groups that did not receive Cilostazol (b).
It was also found that Cilostazol significantly increased hepatocellular proliferation in rats treated with Cilostazol compared to the control group without Cilostazol.

Following major hepatectomy, elevated portal venous perfusion may result in a critical reduction in hepatic artery perfusion. This may cause ischemia-reperfusion injury and acute liver failure in the liver (21-22).

In the current investigation, the groups treated with Cilostazol exhibited significantly elevated SOD activity, while GSH levels, MPO, and AOPPs levels were notably reduced. These observed outcomes can be attributed to the selective inhibition of PDE3 by Cilostazol, which leads to an enhancement in hepatic circulation. Consistent with a prior study, the administration of PDE3 inhibitors has been demonstrated to mitigate hepatic ischemic-reperfusion injury in animal models (23).

Notably, this study pioneers the revelation that the selective PDE3 inhibitor Cilostazol exerts favorable impacts on liver hemodynamics. These findings collectively highlight the potential therapeutic benefits of Cilostazol in the context of liver regeneration and ischemic-reperfusion injury. The widely accepted opinion in ischemia-reperfusion injury following extended hepatic resection is that increased shear stress induces endothelial cell damage during hepatic hyperperfusion. Although liver blood flow increased following hepatectomy in rats treated with Cilostazol, shear stress decreased because of significant vasodilation. This opinion is supported by others who reported reduced portal venous and hepatic arterial resistance despite increased portal venous, hepatic arterial, and hepatic parenchymal blood flow after the inhibition of PDE5 (24).

**Limitations:** The most important limitation of our study is that it is a single-center and retrospective study.

**CONCLUSION**

The current study yields significant insights, demonstrating the beneficial impact of the selective PDE3 inhibitor Cilostazol on histopathological parameters. Notably, Cilostazol exhibited a noteworthy enhancement in hepatocellular proliferation subsequent to extended liver resection. Furthermore, administration of Cilostazol was associated with an increase in the relative weight of the liver, concomitant with a reduction in ischemic-reperfusion injury.

These findings collectively underscore the potential of Cilostazol to contribute to improved treatment strategies for organ donors, as well as to enhance the quality and functionality of liver tissue in the context of liver transplantation.

**Acknowledgments:** None

**Conflict of interest:** The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article. This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

**Author Contributions:** EA, ZE and HE: Concept, Data collection and/or processing, Analysis and/or interpretation, Literature review, EA, ZE and HE: Writing, Revision.

**Ethical approval:** All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and/or with the Helsinki Declaration of 1964 and later versions. Informed consent or substitute for it was obtained from all patients for being included in the study. The ethics committee of this study was approved by the Zonguldak Bülent Ecevit University Medical Faculty Medical and Surgical Research Center with the permission of the Experimental Research Ethics Committee with the number B.30.2.Z.K.Ü.0.20.00.00/33.

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