ABSTRACT

Background: Diabetes mellitus (DM) is a condition which could lead to induce testicular damage due to oxidative stress by hyperglycemic stress. Several therapies have been elucidated to reduce the testicular damage; one of them is Platelet-Rich Plasma (PRP). PRP has known growth factor properties which may serve potential effects in the tissue regeneration. This study aims to determine the role of PRP on testes in streptozotocin-induced diabetic rats by evaluating its impact on testes serum level.

Methods: A randomized clinical trial with control group design has been conducted among 20 rats in this study. The sample of rats was divided into four groups such as control, DM (streptozotocin-induced), DM-Ins (treated with insulin daily), and DM-Ins-PRP (treated with insulin daily and PRP thrice in 2 weeks). Six weeks later, the testosterone level was measured by ELISA, and the right testis was collected for histopathology findings. Data were analyzed using SPSS version 25 for Windows.

Results: The testes weight in the control group was $1.61 \pm 0.37$ grams, followed by DM group ($0.86 \pm 0.22$ grams), DM-Ins group ($1.15 \pm 0.25$ grams), and DM-Ins-PRP group ($1.09 \pm 0.36$ grams). DM reduced testicular weight, seminiferous tubules diameter, the thickness of the epithelium and seminiferous epithelium serum level significantly ($p<0.05$) compared with control. PRP administration increases the thickness of seminiferous epithelium ($p<0.05$) significantly. Besides, it also increased the testicular weight, tubules diameter, lumen diameter, and testosterone serum level, but not significant ($p>0.05$).

Conclusion: The administration of PRP showed a significant role in repairing testicular damage among diabetic rats.

Keywords: Diabetes Mellitus, Platelet-Rich Plasma, Testosterone, Testicular Damage


INTRODUCTION

Diabetes mellitus (DM), which is marked by hyperglycemia, is a metabolic disease that can lead to damage and dysfunction of other organs. The prevalence of DM had reached 415 million people in 2015 and considered to be as high as 642 million people in 2040 worldwide. In Indonesia, the prevalence of DM had reached 8.4 million people in 2000, and it will presumably increase up to 21.3 million people in 2030. Indonesia is also ranked fourth among the world countries which has the highest prevalence of DM. Several studies in humans and animals have shown that DM can impair male reproductive system. DM has several effects on the male reproductive system, such as spermatogenesis impairment, sperm count reduction, sperm movement weakness, and the decline of semen volume and testosterone level. Diabetes can also cause germ cell degeneration, seminiferous tubules diameter, and thickness of seminiferous tubules epithelium reduction.

Platelet-Rich Plasma (PRP) is concentrated plasma containing abundant platelets that are higher compared to the number of normal platelet in the blood. PRP also consists of enriched growth factors that promote angiogenesis, cell proliferation, and differentiation. The growth factors include Vascular Endothelial Growth Factor (VEGF), Platelet-Derived Growth Factor (PDGF), Epidermal growth factor (EGF), Insulin-like Growth Factor (IGF), and Transforming Growth Factor-β (TGF-β). PRP is a simple method product, it also cheap and containing various growth factors that are easy to get.

Research that uses PRP for diabetic wound healing is well known all over the world. Otherwise, the research about the PRP effect on reproductive systems due to diabetes is still limited. Based on those mentioned above, this study aims to determine the impact of PRP administration on testes and testosterone serum levels in Streptozotocin-induced diabetic rats.

METHODS

Animals and Experimental Design

Twenty adult male Wistar rats (2.5 months old, 150-250 g) were purchased from the animal house of the Department of Pharmacology, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada. Rats were put in the cage with room temperature (22°C) in 12h light/12 h dark cycle, 60%
humidity and ad libitum access of food and water. All experiments were conducted as approved by the Medical and Health Research Ethics Committee, Universitas Gadjah Mada.

There were four groups of rats (n=20) in this study such as control, diabetic (DM) with Streptozotocin-induced, diabetic-insulin (DM-Ins) injected daily with 0.9 IU/100 gram bodyweight insulin subcutaneously, and diabetic-insulin-platelet-rich plasma (DM-Ins-PRP) treated with insulin daily and PRP thrice in 2 weeks sub scrotal injection with volume 0.1 ml. To induce diabetes, we injected single intraperitoneal of Streptozotocin (Sigma-Aldrich®, 60 mg/kg) in 0.1 M citrate buffer pH (4.5). Three days after Streptozotocin induction, blood glucose level was checked with GOD PAP enzymatic method, and absorbance was observed using a spectrophotometer at 500 nm to confirm diabetes. Blood glucose level with 200 mg/dl or higher was classified as diabetes. After six weeks of the experiment, all rats were anaesthetized and sacrificed. Before that, blood samples were collected for biochemical examination.

Preparation of Platelet-Rich Plasma (PRP)
A labelled (code or identity of the patient) 10 ml syringe was filled with 1.4 mL citrate buffer. Citrate buffer acts as an anti-clotting agent. From the cubital vein, 10 mL of blood was collected to a labelled syringe. The blood was divided 5 mL each and was filled in two newly syringes. Those syringes were put into a centrifuge machine and rotated at 1200 rpm for 10 minutes. Syringe which was taken from a centrifuge machine and three layers were formed inside, the upper part is plasma fluid, the middle part is the buffy coat, and the lower part is red blood cells. Plasma and buffy coat are transferred by connecting a syringe to a labelled syringe. The blood was divided 5 mL each and was filled in two newly syringes. Those syringes were put into a centrifuge machine and rotated at 1200 rpm for 10 minutes. Syringe which was taken from a centrifuge machine and three layers were formed inside, the upper part is plasma fluid, the middle part is the buffy coat, and the lower part is red blood cells. Plasma and buffy coat are transferred by connecting a syringe containing blood that has been centrifuged to an empty syringe in a vertical position above a syringe containing blood from the centrifuge. The syringe containing plasma and buffy coat were centrifuged for 10 minutes at 3500 rpm. There were two layers in the final result: plasma and deposition of pellet at the bottom. The plasma was partially removed (one-four upper) and then was mixing with pellets.

Measurement of Testicular Weight and Preparation of Histological Testes
After the necropsy, testicles were removed. Left and right testicles were freed from connected tissue around. Each testicle weight was measured and documented in grams (g). The right testicles were immediately fixed using Bouin’s solution. After 24 hours, testicles were trimmed and dehydrated with alcohol and xylene, then embedded in paraffin. The tissue was sliced 5 µm thick using a microtome and mounted on glass objects with albumin coated and then deparaffined with xylol. Finally, tissue sections were stained by the hematoxylin-eosin method to observe histopathological changes under a light microscope.

Histological analysis and Testosterone Measurement
To measure the diameter, lumen diameter, and epithelial thickness of the seminiferous tubules, we use Optilab software, and the value is micrometre (µm). The slide was observed under a light microscope with a magnification of 100x. Seminiferous tubules were chosen randomly with criteria rounded or near rounded tubule. Tubules’ diameter and lumen diameter were determined by the mean of the longest and shortest value. The thickness of seminiferous tubules epithelium was measured using a ruler signer in Optilab Image Raster.

In order to examine the testosterone level, about 2 ml of blood collected from the rat’s orbit sinus was centrifuged for 10 minutes at 3000 rpm. Then, serum was stored in a refrigerator at -80ºC. To measure serum testosterone levels, we used the ELISA method from the manufacturer’s protocol (MBS282195).

Statistical analysis
Statistical Product of Service Solution (SPSS) version 25.0 for Windows was used for the statistical analysis. The data were analyzed using the One-way ANOVA followed by Post-Hoc Test Tukey. If the data is not normally distributed, the Kruskal-Wallis non-parametric statistical test is performed, followed by the Mann-Whitney. The results of p<0.05 were considered as significant.

RESULTS
The testes weight in the control group was 1.61± 0.37 grams, followed by DM group (0.86± 0.22 grams), DM-Ins group (1.15±0.25 grams), and DM-Ins-PRP group (1.09±0.36 grams) (Table 1). The result of this study showed that DM reduced the testes weight compared with the control group significantly (p<0.05) (Table 1).

Figure 1 shows the histological findings in all groups. In control and DM-Ins-PRP group, there was a normal appearance of the tubule, lumen tubule and spermatogenic cells. However, some damage in the tubules, degenerative spermatogenic cells, fewer spermatozoa, or an empty lumen (no spermatozoa) were also found in the DM group and DM-Ins group (Figure 1).
The diameter of seminiferous tubules was 333.69±13.46 µm in the control group, followed by DM (263.02±6.21 µm), DM-Ins (272.28±15.87 µm), and DM-Ins-PRP (291.34±29.72 µm). There was a significant reduction in the diameter of the seminiferous tubules in the DM compared to the control group (p<0.05). In contrast, DM-Ins-PRP did not show a significant reduction in the diameter of the seminiferous tubules (p>0.05) (Table 2). However, the epithelium thickness of the seminiferous tubules was significantly reduced in the DM (50.48±9.50 µm) compared with the control group (98.25±1.42 µm) (p<0.05). Besides, a greater significant thickness of epithelium was found in the DM-Ins-PRP (93.55±11.95 µm) compared with DM (50.48±9.50 µm) group (p<0.05) (Table 2).

Based on the serum testosterone evaluation, it was found that the mean testosterone levels significantly reduced in the DM group (p<0.05) compared with the control group using Kruskal-Wallis & Mann-Whitney test. This reduction was not found in DM-Ins-PRP significantly (p>0.05) (Figure 2).

**DISCUSSION**

In this study, as a type 1 DM inducer animal model Streptozotocin (STZ) could decrease testicular weight. As shown in some previous research that diabetes decreases testicular weight. This decrease linked with tubule atrophy and in the end, testicle atrophy.

Spermatogenesis is a process to produce spermatozoa cells in seminiferous tubules epithelium which consists of spermatogenic and Sertoli cells. Sertoli cells are essential to provide nutrition and growth factors for developing spermatogenic cells. Diabetes changed the histologic structure of testes. The previous study said that diabetes might damage spermatogenesis due to ROS overproduction and decrease endogenous antioxidant. The study has proved that diabetes provoked damage of the histological structure of testicle, these include the irregular arrangement of spermatogenic cells layer, seminiferous tubules impairment, and less to no spermatozoa production in the lumen. The signs of spermatogenesis impairment which is caused by diabetes, are atrophy of tubules and the reduction of spermatogenic cells. The administer of PRP showed repairment of testicular damage, and this can be seen histologically through rearrangement of spermatogenic layer and spermatozoa’s re-produce in the lumen.

In this study, diabetes decreased tubules diameter and tubules epithelium wall significantly, as seen in the previous studies. Compared to the control group, diabetes also increased the lumen

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**Table 1** Testicular weight in rats among groups

<table>
<thead>
<tr>
<th>Variables</th>
<th>Testes Weight (g)(mean±SD)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.61±0.37</td>
<td></td>
</tr>
<tr>
<td>DM</td>
<td>0.86±0.22&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.05&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>DM-Ins</td>
<td>1.15±0.25</td>
<td></td>
</tr>
<tr>
<td>DM-Ins-PRP</td>
<td>1.09±0.36</td>
<td></td>
</tr>
</tbody>
</table>

*Compared with control group with ANOVA-Post Hoc Tukey Test

**Table 2** The comparison of Seminiferous Tubules Diameter (STD), the thickness of epithelium, and seminiferous tubules lumen diameter among groups (mean±SD)

<table>
<thead>
<tr>
<th>Variables (µm)</th>
<th>STD</th>
<th>The thickness of epithelium</th>
<th>ST Lumen Diameter</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>333.69±13.46</td>
<td>98.25±1.42</td>
<td>119.12±9.78</td>
<td></td>
</tr>
<tr>
<td>DM</td>
<td>263.02±6.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>50.48±9.50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>136.35±14.26&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;0.05&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>DM-Ins</td>
<td>272.28±15.87</td>
<td>62.59±6.60</td>
<td>141.85±8.49</td>
<td></td>
</tr>
<tr>
<td>DM-Ins-PRP</td>
<td>291.34±29.72</td>
<td>93.55±11.95&lt;sup&gt;b&lt;/sup&gt;</td>
<td>126.05±11.78&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;0.05&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

ANOVA with the Post-Hoc Tukey test when *compared with control group and *compared with DM group.

**Figure 1** A representative photographs of testicular tissue of rat after H&E (100x) staining in control (A), DM (B), DM-Ins (C), and DM-Ins-PRP (D) groups

**Figure 2** The comparison of mean testosterone levels among groups using Kruskal-Wallis & Mann-Whitney test
diameter of tubules, but not significant. A study conducted by Kianifard D et al. in their study also found the same conclusion. The epithelial walls of DM-Ins-PRP group that was thicker compared to the DM group may indicate that PRP repaired those epithelia. Besides, Dehghani F et al. said that spermatogenic cells (spermatagonia, spermatocyte, spermatid) and the thickness of seminiferous tubules were significantly increased in the PRP administration.

The study proved that diabetes decreases the testosterone levels significantly, same as the previous study. We assumed this mechanism occurred because of the intervention of glucose and gonadotropin level. PRP may improve testosterone levels of diabetic rats but not significant. A DM condition will obstruct steroidogenesis due to no insulin stimulation provided on Leydig cells which decreases the expression of steroidogenesis enzyme (3β-HSD dan stAR).

Nowadays, PRP is used for tissue regeneration because it contains many growth factors that stimulate angiogenesis, cell proliferation and differentiation, and reduce pro-apoptotic Bcl-2 gene expression and inhibit apoptosis. VGEF, one of PRP growth factors induce germ cell proliferation, maintaining their life cycle, and inhibit germ cell apoptosis. Whereas IGF-1 supports spermatogenesis and steroidogenesis. Growth factors in PRP improve cell regeneration and testicular repair damage in streptozocin induced diabetic rats. PRP also suppress caspase-3 expression through activation PI3K/Akt way to limit overproduction of ROS, for the consequences activation of NFkB will also reduce. We conclude PRP has a role in fixing the testicular damage in diabetic rats. More sample is needed for further studies, because in this study, due to limitation, we only perform an experiment with a limited sample.

CONCLUSION
The result of this study showed that DM reduced the testes weight compared with the control group significantly. The DM-Ins-PRP group almost have a similar weight of testes compared with the control group. DM-Ins-PRP group showed a normal appearance of the tubule, lumen tubule, and spermatogenic cells similar to the control group. Also, there was a significant reduction in the diameter and epithelium thickness of the seminiferous tubules in the DM compared to the control group. However, a greater considerable thickness of epithelium was found in the DM-Ins-PRP compared with DM group.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

ETHICAL CLEARANCE
All experiments were conducted as approved by the Medical and Health Research Ethics Committee, Universitas Gadjah Mada, Yogyakarta, Indonesia.

FUNDING
The authors did not receive a grant from any funding agencies.

AUTHOR CONTRIBUTIONS
All of the authors are equally contributed to the study from the conceptual framework, literature search, experimental studies, data gathering, data analysis, until preparing the manuscript for publication.

REFERENCES


