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Immunolocalization of Steroidogenic Enzymes (3β-hydroxysteroid dehydrogenase, 17β-hydroxysteroid dehydrogenase, and P450scc) in Rats with Testicular Dysfunction Treated with Mesenchymal Stem Cells-conditioned Medium

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ABSTRACT

About 60-80 million couples in the world are suffering from infertility disease. Infertility is a major problem in patients coping with chemotherapy. The chemotherapy process can degenerate non-target organs, especially in testes. Infertility in male or testicular dysfunction is caused by the failure of proliferation and differentiation of the spermatogenic cells. Many studies reported that mesenchymal stem cells-conditioned medium promoted regenerative processes. The present study aimed to investigate the effect of mesenchymal stem cells-conditioned medium on the cisplatin-induced testicular dysfunction by examining the immunolocalization of steroidogenic enzymes, such as 3β-hydroxysteroid dehydrogenase, 17β-hydroxysteroid dehydrogenase, and P450scc which are considered as markers of steroid production. All experimental animals were divided into three groups, namely the control group, mesenchymal stem cells-conditioned medium treated group with an injection dose of 0.2 ml/kg body weight (BW, P1), and mesenchymal stem cells-conditioned medium treated group with an injection dose of 0.5 ml/kg BW (P2). Cisplatin was injected into both treated groups to induce testicular dysfunction. The testicular tissues were processed by the paraffin method, then cut to a thickness of 5 µm, followed by immunohistochemical staining. The HSD3B1 immunoreactivities were found only in Leydig cells, and the intensity increased every week after the injection of mesenchymal stem cells-conditioned medium. The variety of weeks and groups was significantly different in the number of immunoreactive cells of HSD3B1. The results indicated a significant difference between one week after the first injection and the one week after the third and fourth injection. The findings showed a significant difference between the treated group with an injection dose of 0.2 ml/kg BW and the control group. The number of immunoreactive cells of HSD3B1 with an injection dose of 0.5 ml/kg BW was greater compared to the group that received an injection dose of 0.2 ml/kg BW. The intensity of HSD3B1 and HSD17B1 increased every week. The p450scc immunoreactive cells were only found in Leydig cells. The intensity of positive cells of p450scc in the treated group with an injection dose of 0.5 ml/kg BW was more intense, compared to the treated group with an injection dose of 0.2 ml/kg BW. The results of the current study showed that the injection of mesenchymal stem cells-conditioned medium can improve the regeneration of spermatogenic cells, and recover spermatogenesis proved by positive cells of HSD3B1, HSD17B1, and p450scc as markers of steroid production.

Keywords: Cisplatin, HSD17B1, HSD3B1, Mesenchymal stem cells-conditioned medium, P450scc, Testicular dysfunction

INTRODUCTION

About 60-80 million couples in the world are suffering from an infertility disease, and about 40-50% of the infertility is caused by male factors. Infertility is an extremely common physical disorder in adults, which occurs due to trauma, infections, tumor growth, and radiation therapy in patients coping with chemotherapy (Zhang et al., 2017). The chemotherapy process can degenerate non-target organs, especially in testes, that decrease the quality and quantity of sperm. Infertility in male or testicular dysfunction is caused by the failure of proliferation and differentiation of the spermatogenic cells. Testicular dysfunction in an animal model can be induced by anticancer drugs, such as busulfan, cyclophosphamide, doxorubicin, procarbazine, or cisplatin (Atessahin et al., 2006). Cisplatin is an anticancer drug potential; however, a high dose of cisplatin is extremely toxic for testicles. Some researchers reported that the administration of antioxidants, such as lycopene, royal jelly, curcumin, melatonin, Vitamin C, montelukast, resveratrol, selenium, and amifostine, can improve the degeneration of testis (Beytur et al., 2012; Reddy et al., 2016; Simsek et al., 2016).

Mesenchymal Stem Cells (MSCs) have been used widely in the treatment of organ dysfunctions, such as testicular dysfunction. Many studies have reported the improvement of androgen deficiency after stem cell therapy. Human Umbilical Mesenchymal Stem Cells (HUMSCs) can increase testosterone levels, and Leydig-like cells are functioning

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well in hypogonadism models (Zhang et al., 2017). Transplanted rat adipose-derived MCSs into D-galactose-treated aging rats can improve testicular function by being differentiated into 3-β-Hydroxysteroid Dehydrogenase (HSD3B1) positive Leydig-like cells (Yang et al., 2015). Tamadon et al. (2015) reported that transplanted Bone Marrow-Mesenchymal Stem Cells (BM-MSCs) could successfully induce spermatogenesis in seminiferous tubules of azoospermia hamsters. Mesenchymal stem cells-conditioned medium or secretome is a factor secreted by stem cells containing growth factors and cytokines. Many studies reported that mesenchymal stem cells-conditioned medium promotes regenerative processes, such as healing diabetes mellitus type 1 by improving the pancreatic beta cells (Nugroho et al., 2016), and regenerating the healing burns (Padeta et al., 2017). It also regenerates the incise wound healing (Kusindarta et al., 2016). The present study aimed to investigate the effect of mesenchymal stem cells-conditioned medium in cisplatin-induced testicular dysfunction by examining the immunolocalization of HSD3B1, 17β-Hydroxysteroid Dehydrogenase (HSD17B1), and P450 Side-Chain Cleavage enzyme (P450scc).

MATERIALS AND METHODS

Ethical approval

The present study was approved by Ethical Clearance from Universitas Gadjah Mada, Yogyakarta, Indonesia with number 00035/04/LPPT/V/2017

Experimental animals

A total number of 36 healthy male Wistar rats (Rattus norvegicus) aged three-month-old, with an average weight of about 300 grams, were randomly divided into three groups. The groups included the control group, and the mesenchymal stem cells-conditioned medium treated groups with an injection dose of 0.2 ml/kg BW (P1), and the mesenchymal stem cells-conditioned medium treated groups with an injection dose of 0.5 ml/kg BW (P2). The control group was not injected with cisplatin, but injected with 0.9% physiological NaCl. The P1 and P2 groups were injected with cisplatin to induce testicular dysfunction, and continued with an injection dose of 0.2 ml/kg BW and 0.5 ml/kg BW of mesenchymal stem cells-conditioned medium. The rats in the treated groups were injected with Cisplatin (PT Dankos Farma, Kalbe Company, Indonesia) at a dose of three mg/kg BW three times at three-day intervals (Reddy et al., 2016; Prihatno et al., 2018). The mesenchymal stem cells-conditioned medium was injected one time a week for four times after all doses of cisplatin were injected.

Sample collection

Testicular tissues were collected one week after the first, second, third, and fourth injection of mesenchymal stem cells-conditioned medium. Testicular tissues were fixed in Bouin's solution for 24 hours, embedded in paraffin, sectioned to a thickness of 5 µm, and stained with the immunological method (Prihatno et al., 2020) to visualize the immunoreactivity of HSD3B1, HSD17B1, and P450scc.

Immunohistochemical staining

The first step of immunohistochemical staining was deparaffinization with xylene. Then, the slides were rehydrated with ethanol, and rinsed in running water for 5 minutes. Distillate water was placed in the microwave for 20 minutes for pre-heating the antigen retrieval, and then the slides were immersed in pre-heating distillate water for 10 minutes for antigen retrieval. Endogenous peroxide activity was blocked by incubating the slides in H_2O_2 3% in absolute methanol (1 mL H_2O_2 30%, 9 mL absolute methanol) for 30 minutes at room temperature. The slides were washed in Phosphate Buffer Saline (PBS) for five minutes, then repeated three times. Immunoreactivity was visualized with rabbit Diaminobenzidine (DAB) Detection IHC Kit (Fine Test, Wuhan Fine Biotech Co., Ltd., China) (Prihatno et al., 2020). After that, the slides were incubated overnight at 4^0C with HSD3B1 polyclonal antibody (1: 200, MBS2530192, Mybiosource, USA), HSD17B1 polyclonal antibody (1:400, A10839, ABclonal, USA), and Cytochrome P450scc Enzyme polyclonal antibody (1:500, AB1244, MilliporeSigma, USA). After that, the slides were washed again in PBS for five minutes, then it was repeated three times, and they were incubated with poly-HRP Goat Anti-Rabbit IgG for 60 minutes, and then for the last time, the slides were washed again in PBS three times for five minutes. Sites of immunoreactivity were visualized with DAB Chromogen. Harris hematoxylin was used for counter-staining the slides, followed by rinsing the slides with running water for 10 minutes. Lastly, the slides were dehydrated, cleared, and mounted. A light microscope was used to examine the stained slides, and Optilab® camera was used to take a photomicrograph.

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Statistical analysis

The number of immunoreactive cells of HSD3B1 was analyzed statistically using the Analysis of Variance (ANOVA) test followed by Duncan's post hoc test with software IBM SPSS Statistic 22. In this study, $p < 0.05$ was considered significant.

RESULTS

The immunoreactivity of HSD3B1 was detected in Leydig cells, and the intensity increased every week (Figures 1 and 2). The intensity of both treated groups, P1 and P2, on one week after the first injection, the immunoreactivity showed weak intensity (Figures 1-A and 2-A). One week after the second injection, the immunoreactivity of treated groups, both P1 and P2, showed no difference compared to the control group. One week after the third and the fourth injections, the intensity of both treated groups was stronger compared to the control group.

The obtained results of different time periods (one week after the first, second, third, and fourth injections) indicated a significantly different number of immunoreactive cells of HSD3B1 ($p < 0.05$). The investigated groups (P1, P2, and the control group) were significantly different regarding the number of immunoreactive cells of HSD3B1 ($p < 0.05$, Table 1). The results revealed a significant difference between the obtained results of one week after the first injection and one week after the third and fourth injection. However, one week after the first injection was not significantly different compared to one week after the second injection (Table 2). The statistical analysis of groups showed a significant difference between the P1 and control groups ($p < 0.05$). However, the P1 group was not significant in the number of immunoreactive cells of HSD3B1 compared to the P2 group. The control group was not significantly different in the number of immunoreactive cells of HSD3B1 compared to the P2 group (Table 3). The number of immunoreactive cells of HSD3B1 in the P2 group was greater compared to the P1 group (Table 3).

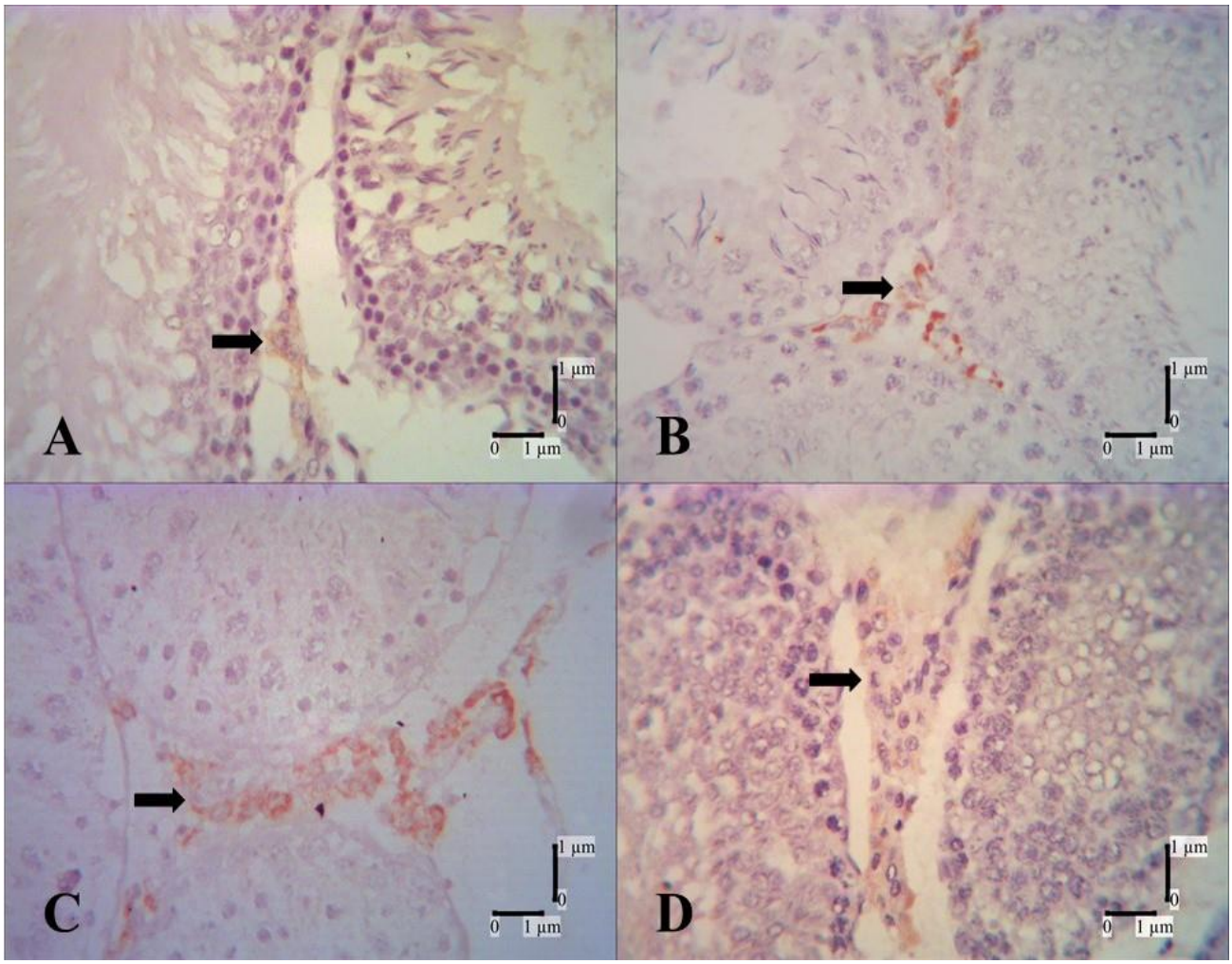

Figure 1. Immunoreactive cells of HSD3B1 with injection dose of 0.2 ml/kg BW mesenchymal stem cells-conditioned medium. **A:** One week after the first injection, **B:** One week after the second injection, **C:** One week after the third injection, **D:** the control group; black arrow: Immunoreactive cells of HSD3B1

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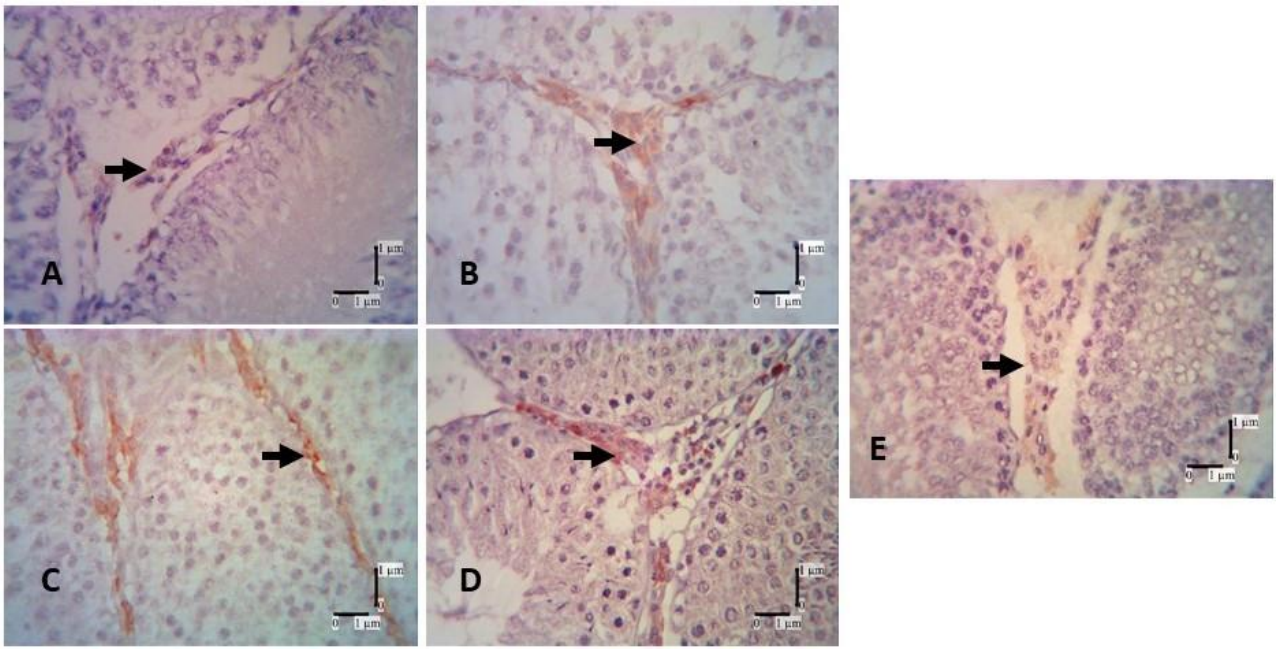

Figure 2. Immunoreactive cells of HSD3B1 with injection dose of 0.5 ml/kg BW mesenchymal stem cells-conditioned medium. **A:** One week after the first injection, **B:** One week after the second injection, **C:** One week after the third injection, **D:** One week after the fourth injection, **E:** Control group, black arrow: Immunoreactive cells of HSD3B1

Table 1. Analysis of variance at the number of immunoreactive cells of HSD3B1

Source	Type III Sum of Squares	df	Mean Square	F	p value
Corrected model	7115.575[a]	5	1423.115	9.591	0.000
Intercept	37238.450	1	37238.450	250.973	0.000
Weeks	6189.083	3	2063.028	13.904	0.000
Groups	2551.800	2	1275.900	8.599	0.001
Error	5044.800	34	148.376		
Total	55391.000	40			
Corrected Total	12160.375	39			

[a.] R Squared = 0.585 (Adjusted R Squared = 0.524). R Squared (R^2) showed that the variety of weeks effects on a variety of groups was 58,5%. The significance level less than or equal to 0.05 was considered statistically significant ($p < 0.05$).

Table 2. The mean of immunoreactive cells of HSD3B1 one week after the first, second, third, and fourth injection of mesenchymal stem cells-conditioned medium, and in the control group in rats with testicular dysfunction

Week	Mean
One week after the first injection	21.80[a]
One week after the second injection	30.90[a]
One week after the third injection	41.70[bc]
One week after the fourth injection	52.40[c]

[a,b,c] Means within a column with different superscripts differ significantly ($p < 0.05$).

Table 3. The mean of immunoreactive cells of HSD3B1 in the P1, P2, and control group in rats with testicular dysfunction

Group	Mean
The P1 group	26.93[a]
The P2 group	35.55[ab]
Control group	40.00[b]

The P1 group: mesenchymal stem cells-conditioned medium treated group with an injection dose of 0.2 ml/kg BW; The P2 group: mesenchymal stem cells-conditioned medium treated group with an injection dose of 0.5 ml/kg BW; [a,b] Means within a column with different superscripts differ significantly ($p < 0.05$).

The immunoreactivity of HSD17B1 was detected in Sertoli cells, Leydig cells, and spermatids. The intensity was increased every week. At first, the intensity of immunoreactive cells detected in spermatids and Sertoli were weak at one week after the first injection in the P1 group. The spermatogenic cells in one week after the first and the second injections were still not complete compared to one week after the third injection (Figure 3). The intensity of HSD17B1 increased in the P2 group (Figure 4). One week after the second injection of the P2 group, the spermatogenic cells improved similar to the ones in the control group. One week after the first injection, the intensity was weak. One week after the second and the third injections, the intensity was strong, and one week after the fourth injection, it was very strong. The spermatogenic cells in the group with cisplatin-induced testicular dysfunction were destroyed and lost

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(Figure 5). The immunoreactivity of p450scc was detected in Leydig cells. The immunoreactivity in the P2 group was increased one week after the second injection of mesenchymal stem cells-conditioned medium. One week after the first injection, the intensity was weak. However, one week after the third and the fourth injections was strong. The number of positive cells increased every week (Figure 6). The immunoreactivity of p450scc was detected weak one week after the first injection of 0.2 ml/kg BW mesenchymal stem cells-conditioned medium. Although in one week after the second and the third injections, it was strong (Figure 7). The immunoreactivity of p450scc on the control group was detected in Leydig cells with strong intensity (Figure 8-B). The immunoreactivity of p450scc in the group with cisplatin-induced testicular dysfunction was not expressed. A lot of spermatogenic cells have been damaged (Figure 8-A).

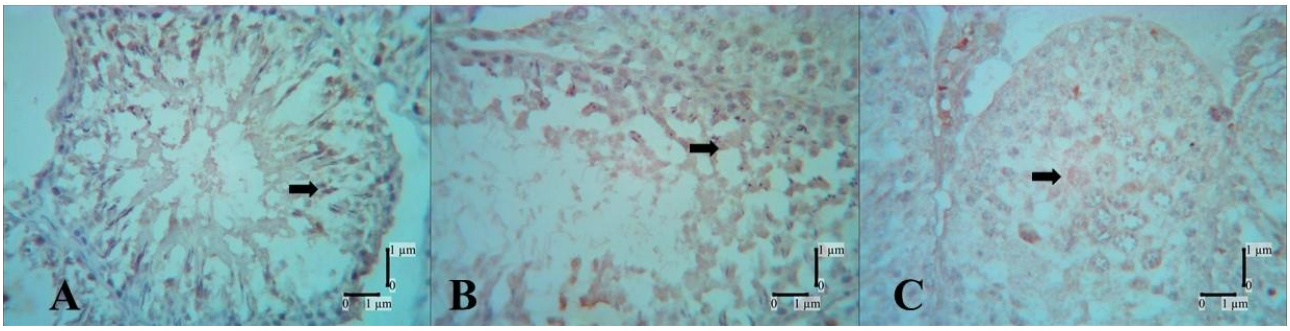

Figure 3. Immunoreactive cells of HSD17B1 of the treated group with an injection dose of 0.2 ml/kg BW of mesenchymal stem cells-conditioned medium. Immunoreactive cells were detected in Sertoli cells and spermatids with a weak intensity one week after the first injection dose of 0.2 ml/kg BW mesenchymal stem cells-conditioned medium. **A:** One week after the first injection, **B:** One week after the second injection, **C:** One week after the third injection, black arrow: IMMUNOREACTIVE cells of HSD17B1

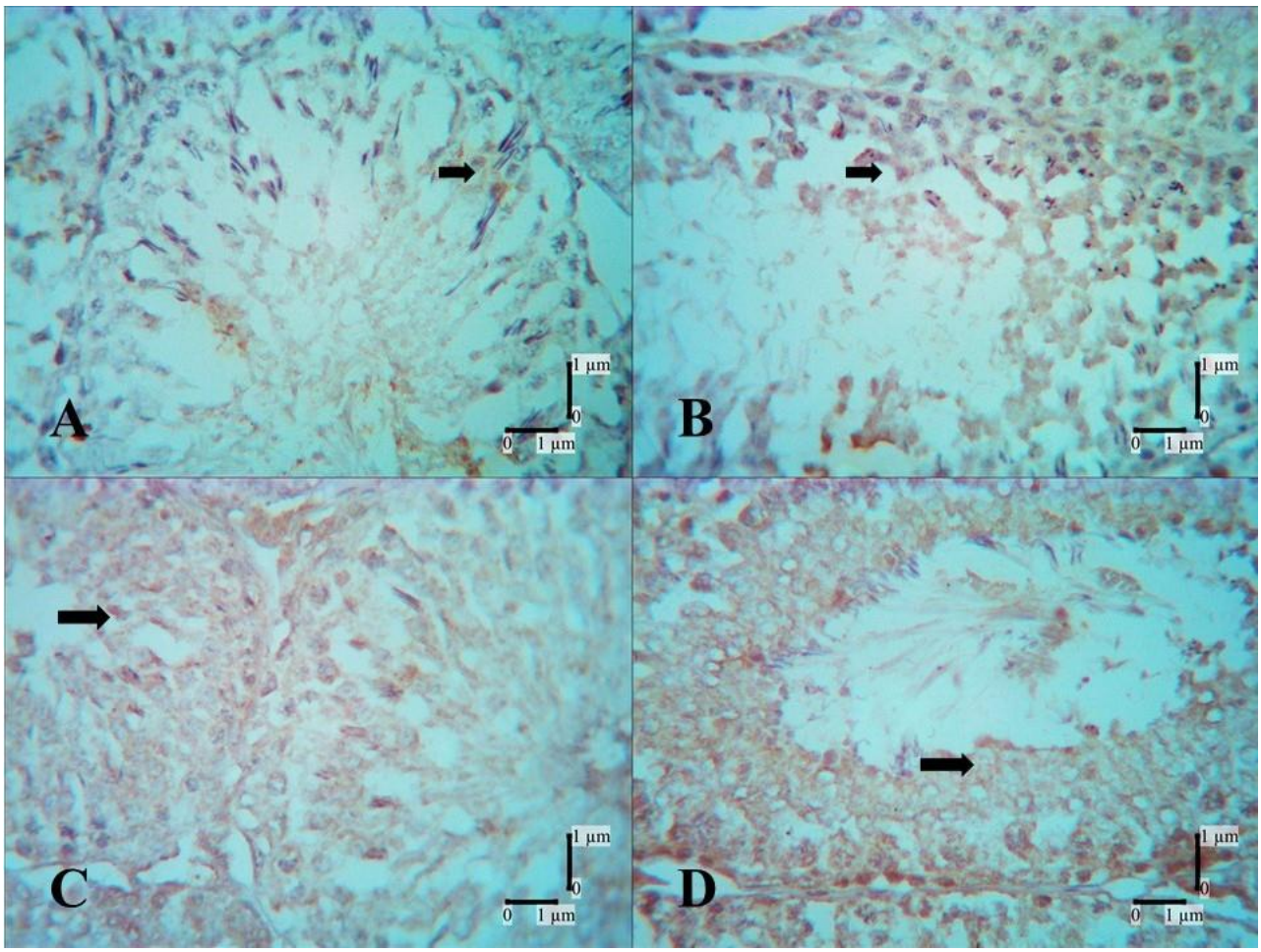

Figure 4. Immunoreactive cells of HSD17B1 in the treated group with an injection dose of 0.5 ml/kg BW of mesenchymal stem cells-conditioned medium treated group. **A:** One week after the first injection, **B:** One week after the second injection, **C:** One week after the third injection, **D:** One week after the fourth injection, black arrow: Immunoreactive cells of HSD17B1

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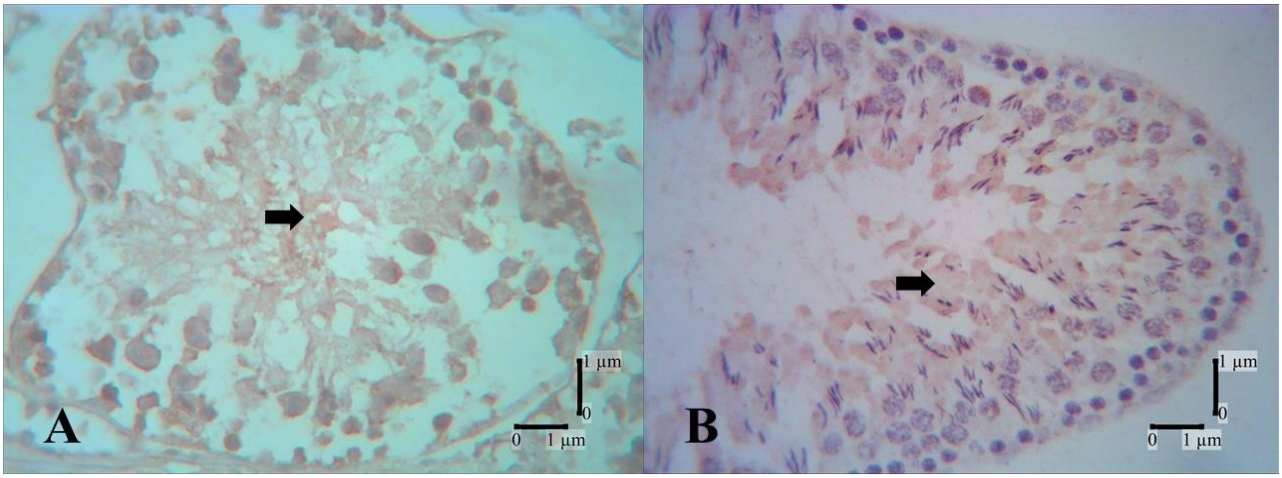

Figure 5. Immunoreactive cells of HSD17B1 in the cisplatin-induced group with the testicular dysfunction and the control group. **A:** Cisplatin-induced testicular dysfunction, **B:** Control group**,** black arrow: Immunoreactive cells of HSD17B1

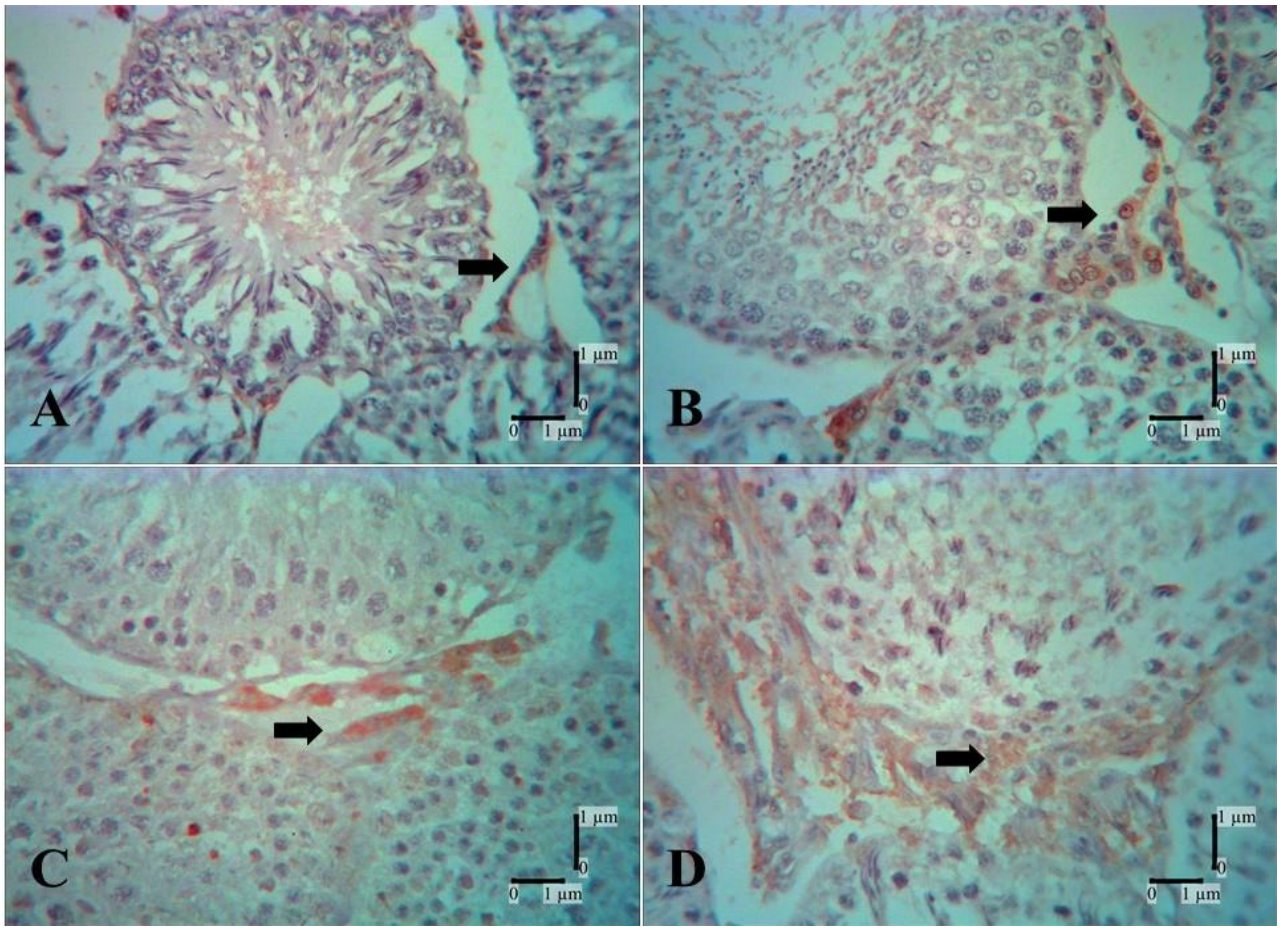

Figure 6. Immunoreactive cells of p450scc in the treated group with an injection dose of 0.5 ml/kg BW of mesenchymal stem cells-conditioned medium. **A:** One week after the first injection, **B:** One week after the second injection, **C:** One week after the third injection, **D:** One week after the fourth injection, black arrow: Immunoreactive cells of p450scc.

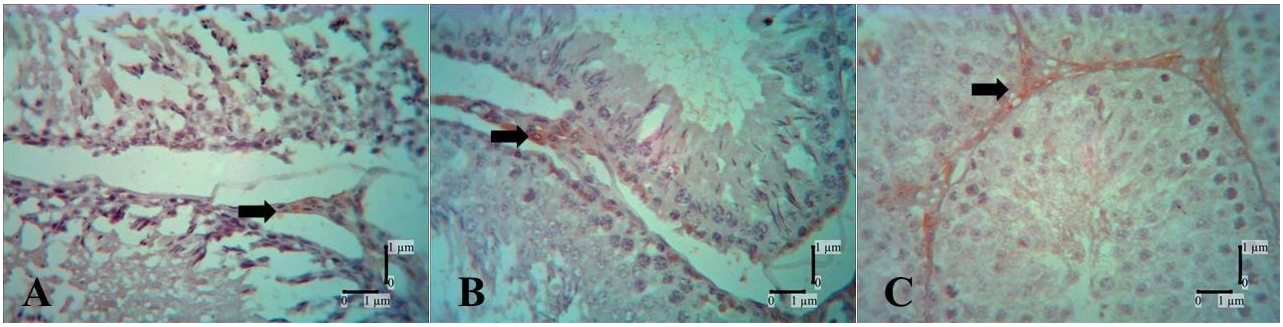

Figure 7. Immunoreactive cells of p450scc in the treated group with an injection dose of 0.2 ml/kg BW of mesenchymal stem cells-conditioned medium. **A:** One week after the first injection, **B:** One week after the second injection, **C:** One week after the third injection, black arrow: Immunoreactive cells of p450scc.

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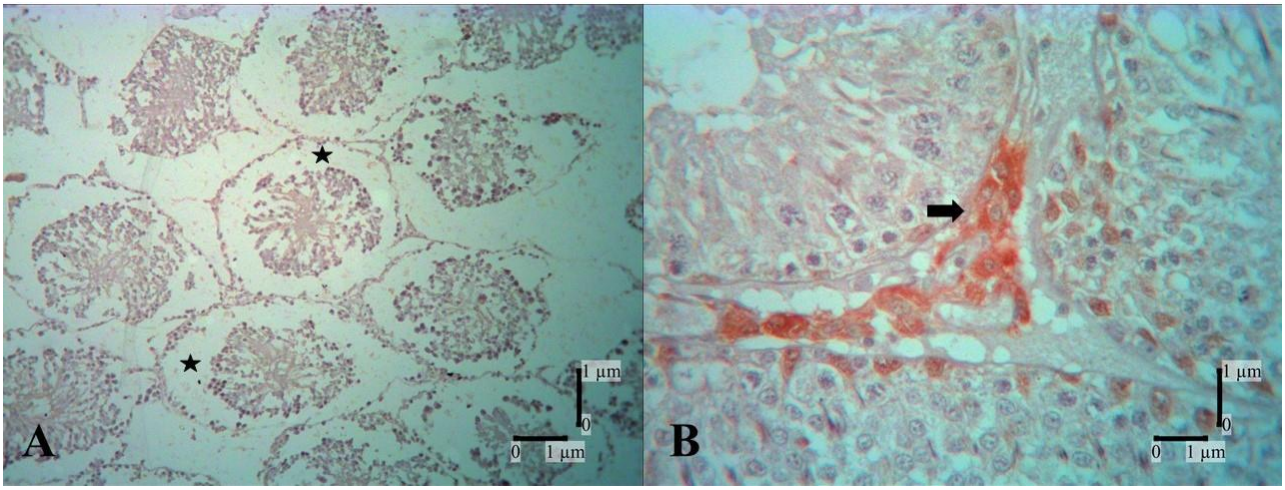

Figure 8. Immunoreactive cells of p450scc in the cisplatin-induced group with the testicular dysfunction and the control group. **A:** Cisplatin-induced testicular dysfunction group. **B:** Control group. Black arrow: Immunoreactive cells of p450scc, black star: Damage of seminiferous tubules.

DISCUSSION

Steroidogenesis involves multiple processes for the biosynthesis of steroid hormones (testosterone) from cholesterol in Leydig cells. Steroid hormones regulate sexual development and are essential for physiological functions. In males, the Leydig cells are considered important for producing the testosterone hormone that is critical for spermatogenesis and maintenance of secondary sexual functions (Wang et al., 2017). Steroidogenesis depends on the steroidogenic enzyme, including P450scc, HSD3B1, HSD17B1, 17a-hydroxylase/C17, 21 lyases (P450c17), and aromatase cytochrome P450 (P450arom) as markers of steroid production (Kobayashi et al., 1998).

The HSD3B1 is a key enzyme necessary for the synthesis of testosterone in Leydig cells in rats. This enzyme has a major catalytic activity in changing pregnenolone, 17-OH-pregnenolone, dehydroepiandrosterone (DHEA), and androstenediol. The deficiency of HSD3B1 affects the gonads (testes in males) and adrenal glands, which reduces the production of the testosterone hormone. The HSD17B1 enzyme is involved in the local regulation of sex steroids (Gunnarsson et al., 2005). The HSD17B1 is involved in the final step of the biosynthesis of testosterone, which catalyzes androstenedione to testosterone hormone (Baker et al., 1997). In immunohistochemistry methods, the immunoreactivity expresses in Leydig cells of interstitial cells of testes (Hanukoglu, 1992). The P450scc catalyzes three chemical reactions, 20a-hydroxylase, 22- hydroxylase, and cuts the side chain of cholesterol into pregnenolone. In the testis, P450scc is detected in Leydig cells (Hanukoglu, 1992; Ulloa-Aguirre and Conn, 2014).

The present study showed that the injection of a mesenchymal stem cells-conditioned medium in rats with cisplatin-induced testicular dysfunction promoted the recovery of testicular degeneration. The immunohistochemistry method detected the expression of HSD3B1, HSD17B1, and p450scc as markers of steroidogenesis. The immunoreactivity of HSD3B1, HSD17B1, and p450scc was shown in Leydig cells, and the intensity increased every week after the injections of mesenchymal stem cells-condition medium. In the immunohistochemical staining, localization of HSD3B1 and HSD17B1 was on steroidogenic cells, selectively expressed in Leydig cells in the interstitial cells of the testis, and it was not found in seminiferous tubules. The upregulation of cellular factors and the testicular microenvironment can occur along with the regeneration and differentiation of Leydig cells via secretion of cellular factors such as epidermal growth factors, insulin-like growth factors-1, and platelet-derived growth factors, that contain in HUMSCs (Lin et al., 1998; Odeh et al., 2014; Zhanget al., 2017). The current study reported the p450scc positive in Leydig cells in both treated groups. The steroidogenic process maybe was developed to make steroid hormones. Stem cell therapy may help to reduce the effect of cancer therapy on testicular dysfunction in the patients coping with cancer (Tamadon et al., 2015). Transplantation of mesenchymal stem cells induced spermatogenesis in azoospermic models (Moreno et al., 2015); differentiated into germ cells, Sertoli cells, and Leydig cells (Lue et al., 2007; Monsefi et al., 2013). Mesenchymal Stem Cells (MSCs) might be able to recover the regeneration of testicular dysfunction by three mechanisms, firstly, MSCs may transform into target cells via appropriate induction conditions. Secondly, growth factors are secreted by MSCs to repair the cell function, and finally, MSCs are incorporated with the endogenous cells repairing the injured tissue function (Mansour et al., 2012; Leatherman, 2013; Tamadon et al., 2015).

Mesenchymal stem cells-conditioned mediums or secretome are factors secreted by stem cells containing growth factors and cytokines, such as vascular endothelial and epidermal growth factors, insulin growth factor, interleukin, tumor necrosis factor, and interferons, that potentially modulates cellular processes, including cell proliferation, differentiation, immunomodulation, migration, angiogenesis, and survival. Mesenchymal stem cells have been used in

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tissue repair, and they can differentiate into Leydig-like cells in vitro (Yazawa et al., 2006). These cellular factors can then promote the regeneration of Leydig cells, and enhance their functions (Ricci et al, 2012; Odeh et al., 2014).

CONCLUSION

The results of the present study showed that the injection of a mesenchymal stem cells-conditioned medium can improve the regeneration of spermatogenic cells, and recover spermatogenesis proved by positive cells of HSD3B1, HSD17B1, and p450scc as markers of steroid production.

DECLARATIONS

Authors' contribution

Linda M. Khasanah contributed to data analysis and the write-up of the manuscript, Teguh Budipitojo created the concepts and designed the experiments, interpreted the data, read and approved the final manuscript. Yuda H. Fibrianto developed the concepts and designed the experiment, and produced secretome. All authors approved the final manuscript and checked the ethical issues.

Competing interests

The authors declared no conflictS of interest.

Acknowledgments

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Ethical considerations

Ethical issues (including plagiarism, consent to publish, misconduct, data fabrication and/or falsification, double publication and/or submission, and redundancy) have been checked by the authors.

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