

The Effect of Bone Marrow Transplantation on Oocyte-Granulosa Cell Interaction and Follicular Development of Cisplatin-Induced Ovarian Failure in Rat

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ABSTRACT

Introduction: Chemotherapy has cytotoxic effect that induces follicular damage and abnormal folliculogenesis leads to ovarian failure. Two crucial growth factors in abnormal folliculogenesis, Growth Differentiation Factor-9 (GDF-9) and Kit-Ligand, will be disrupted and affect follicular development. In this study, we evaluate whether bone marrow transplantation (BMT) has a role on oocyte-granulosa cell interaction by analyzing GDF-9 and Kit-Ligand expressions and also follicular development by analyzing primordial, primary, secondary and graafian follicles of cisplatin-induced ovarian failure in rat. Material and method: Forty eight rats were divided into three groups: control, cisplatin and cisplatin + BMT. Ovarian failure was induced by administration of intraperitoneal cisplatin 5 mg/kg body weight for 1 week. BMT 2×10^7 cells were injected through rat tail vein after cisplatin administration. Bone marrow was isolated from rat femur and characterized by CD44(+), CD45(-), CD105(+). Immunohistochemistry examinations for ovarian GDF-9, Kit-Ligand and follicle development evaluation were performed after 2 weeks of BMT injection. Results: The expressions of Kit-ligand among three groups by ANOVA were significant different ($p=0.00$), whereas by Post Hoc: cisplatin group lower than control group ($p=0.00$); cisplatin + BMT group higher than cisplatin group ($p=0.00$); and no significant different between control group and cisplatin + BMT group ($p=0.955$). The expressions of GDF-9 by Kruskal Wallis showed significant different ($p=0.00$) among three groups whereas cisplatin + BMT group higher than cisplatin group and control group. In cisplatin + BMT group the number of primordial, primary, secondary and graafian follicles were higher than those in cisplatin group; but were lower than those in control group ($p=0.000$). Positive Paul Kart Horan (PKH) labeling was seen in cisplatin + BMT group only. Conclusion: In cisplatin-induced ovarian failure in rat, bone marrow transplantation may improve oocytegranulosa cell interaction and follicular development.

Key words: Bone Marrow, GDF-9, Kit-ligand, follicle, Ovarian failure

INTRODUCTION

Chemotherapy is one part of cancer therapy with the aim of suppressing the growth of the disease and improves the quality of life of the patients (WHO, 2008). The use of chemotherapy has significantly increased the cure rate in cancer patients in young age (Su, 2011). However, some side effects, such as ovarian failure and infertility, may occur. Ovarian failure is a consequence of chemotherapy in cancer patients due to cytotoxic effects that will damage the granulosa cells and induces acute damage in ovarian follicle growth so that folliculogenesis disruption may take place (Melrow and Nugent, 2001; Dolmans et al., 2005). Various methods of treatment have been made to overcome ovarian failure due to chemotherapy, such as stem cell therapy. However, to date the effect of stem cell transplantation to repair folliculogenesis disruption in ovarian failure remains a debate.

Folliculogenesis is a process of ovarian follicles growth and development, which is the interaction of oocyte and granulosa cells with

resulting in mature and fertilizable oocytes. There are two crucial growth factors that play a role in the interaction of oocyte and granulosa cells, namely Growth Differentiation Factor-9 (GDF-9) derived from oocyte and Kit-Ligand produced from granulosa cells (Rajkovic et al., 2006; Joyce et al., 2000; Hendarto, 2012). In abnormal folliculogenesis, the interaction of both growth factors could disturb the growth of follicles in the ovaries. The use of bone marrow mesenchymal stem cells, which have the ability to regenerate itself and differentiate into other tissue cells, has been tried for regeneration therapy of various diseases. Our current study aimed to evaluate the effect of allogeneic bone marrow transplantation on the oocyte-granulosa cell interaction by analyzing the expression of GDF-9 and Kit-Ligand and the description of follicular development by analyzing the number of primordial, primary, secondary, and graafian follicles in cisplatin-induced ovarian failure in rat.

MATERIALS AND METHODS

Animal

This study was an experimental laboratory study with a double-blind randomized design. The population in this study were female rats (wistar strain *Rattus norvegicus*) obtained from the laboratory animal unit of the Faculty of Veterinary Medicine, Airlangga University. Before starting, female rats were caged for a week at room temperature and given with ad libitum

diet. Forty-eight rats that met the inclusion and exclusion criteria with homogeneous characteristics in terms of mean age and body weight were divided into three groups. The first group was the control group, injected with 0.9% NaCl intraperitoneum, the second group was the group of rat models with ovarian failure, administered with cisplatin 5 mg/kg

intraperitoneally for 1 week, and the third group was the group of rat models with ovarian failure, which, after receiving cisplatin, were administered

with bone marrow stem cell transplantation (BMT) for 2 weeks.

Isolation, culture and transplantation of bone marrow stem cells.

Bone marrow aspirates were collected from donor rat femoral bone with a local anesthetic. Each aspirate was mixed and coated with Ficoll and then centrifugated in 1600 rpm for 15 minutes. The 'huffy coat' located on Ficoll-PBS was collected with a Pasteur pipette. Cells were placed on 5 or 10 cm² plate, followed by cell incubation at 37°C with humidity of 5-% CO₂ for 24 hours.

Examination with microscope was done every day and every 3 days the cells were washed with 5 or 10 ml PBS and 10 ml. CCM was added until the cells were became confluent between 60-80%.

Bone marrow as many as 2×10^7 cells were injected through recipient rat tail intravenously 1 week after intraperitoneal cisplatin administration (Rantam et al., 2009).

GDF-9, Kit-ligand and follicular development evaluation

Two weeks after bone marrow transplantation the ovarian expression of GDF-9 and Kit-Ligand were evaluated with immunohistochemical method by quantity of cells that express in ten views. Anti GDF-9 and Kit-Ligand antibody was purchased from Abcam.' Primordial, primary, secondary, and graafian follicles development were

evaluated using haematoxillineosin staining method in three groups of rats above. All data were analyzed using statistical SPSS/PC computer program for windows. All three groups' data were compared using the ANOVA test. The differences are considered to be statistically significant if $p < 0,05$.

RESULT

This research was conducted between May and August 2019 in Experimental Animal Cage, Veterinary Pathology Laboratory, Faculty of Veterinary Medicine, University of Airlangga Surabaya Two weeks after BMT administration, the rats were sacrificed and subsequently the

ovarian tissue was evaluated. Previously, characterization had been carried out to bone marrow stem cell preparation with the CD44(+), CD45(-), CD-105 (+) which indicated mesenchymal stem cells

GDF-9 expression

Immunohistochemical analysis of the three groups of rat ovaries showed a positive signal for GDF-9 protein. Analysis of GDF-9

expression in 'all three groups were as follows: control (14.53 ± 1.42); cisplatin (5.33 ± 1.76) and cisplatin + BMT group (15.91 ± 0.69), there were

significant differences in the three groups above ($p=0.00$) by Kruskal Wallis. The count was in accordance with the results of immunohistochemical staining images marked in brown colour on the immune reactive cells that

Kit-Ligand expression

Immunohistochemical analysis in all three groups of rat ovaries also showed a positive signal for Kit-Ligand protein. Analyses of Kit-Ligand expression in all three groups were as follows: control (20.22 ± 2.14), cisplatin (12.27 ± 2.88) and cisplatin + BMT group (20.26 ± 1.14). There were significant differences in the three groups above ($p=0.00$) by ANOVA; whereas by Post Hoc: cisplatin group lower than control group ($p=0.00$); cisplatin + BMT group higher

Follicular development

The process of follicular development is characterized by the development of primordial primary, secondary, graafian follicles. In this study the cisplatin group showed the number of primordial (4.31 ± 1.19), primary (3.81 ± 1.22), secondary (2.87 ± 0.95), and graafian follicles (0.37 ± 0.69) were lower than those in control group (6.12 ± 1.20), (4.93 ± 1.61), (4.25 ± 0.77) and (5.81 ± 1.37) ($p=0.000$).

DISCUSSION

As we all know there is dependence interaction between oocyte and granulosa cells. Oocytes secrete factors that regulate the development of granulosa cells surrounding the oocytes in the

express the protein GDF-9. The lowest expression of GDF-9 was found in cisplatin administration, whereas the highest expression was found in cisplatin + BMT group (Figure 1).

than cisplatin group ($p=0.00$); and no significant different between control group and cisplatin + BMT group ($p=0.955$). The results above were in accordance with immunohistochemical staining images marked in brown colour on the immunoreactive cells that express Kit-Ligand protein. The lowest expression of Kit-Ligand was found in cisplatin administration, whereas the highest expression was found in in cisplatin + BMT group (Figure 2).

In cisplatin + BMT group, the number of primordial (5.31 ± 1.30), primary (4.37 ± 0.88), secondary (3.62 ± 0.71) and graafian follicle (2.75 ± 0.85) were higher than those in cisplatin and control group (Figure 3). In this study, *Paul Karl Horan* (PKH) cell labeling was also conducted. Subsequently, signal identification was carried out after BMT, and positive PKH labeling was seen in cisplatin + BMT group, while negative result was found in cisplatin group (Figure 4).

ovarian follicle. Granulosa cells secrete Kit-Ligand to regulate oocyte growth and development. The main candidate molecules secreted by the oocyte is



Figure 1: Immunohistochemical staining for GDF-9 in rat ovarium of (A) control, (B) cisplatin and (C) cisplatin+BMT groups show the immunoreactive cells with brown colour.

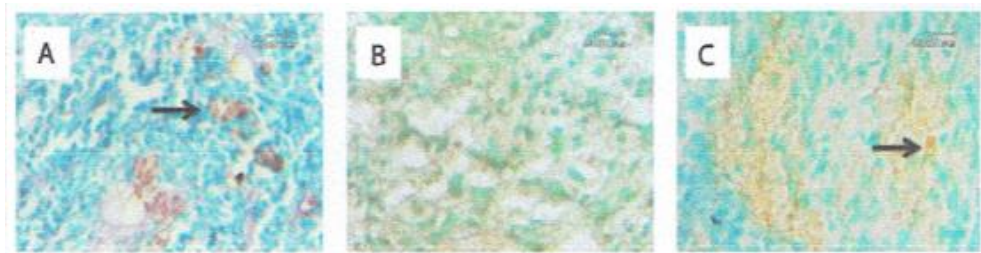


Figure 2: Immunohistochemical staining for Kit Ligand in rat ovarium (A) control, (B) cisplatin and (C) cisplatin+BMT groups show the immunoreactive cells with yellow to brown colour.

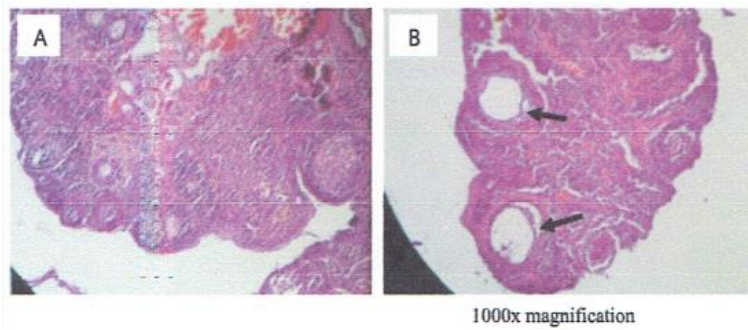


Figure 3: Haematoxylin eosin staining for follicular development in (A) cisplatin and (B) cisplatin+BMT group. Graafian have seen in cisplatin+BMT group but not in cisplatin group.

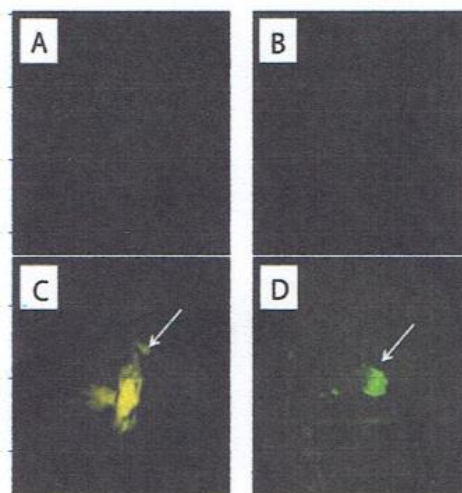


Figure 4: PKH for fluorescent cell labeling in (A&B) cisplatin and (C&D) cisplatin+BMT group

GDF-9, a growth factor from the family TGF- β produced by oocytes (Joyce et al., 2000). Filho suggested that GDF-9 is a crucial factor in folliculogenesis and fertility, and it was proved that the expression of GDF-9 decreases in women with polycystic ovary indicated by the disturbance oocyte growth and differentiation (Teixeira et al., 2001). This study revealed the difference in the expression of GDF-9 in the three groups. The expression of GDF-9 in the cisplatin group (5.33 ± 1.76) was lower compared to that in control group (14.53 ± 1.42), which means that there has been a disturbance in folliculogenesis due to disrupted production of GDF-9, allegedly due to apoptosis in the oocyte resulting from cisplatin chemotherapy.

Cisplatin is a chemotherapy drug from the class of alkylating agents from platinum compounds used for the treatment of ovarian, breast and bladder cancers. Once administered, cisplatin will diffuse inside the cell which then binds to DNA, thereby preventing DNA transcription and replication. Cis-DDP (Diammine Dichloro Platinum) binding on g (genomic) DNA contained in the nucleus is responsible for the mechanism and will trigger cell death/apoptosis (Turkyilmaz et al., 2008). In group 3 that received BMT GDF-9 expression was higher (15.91 ± 0.69) than cisplatin group (5.33 ± 1.76). This result suggests that BMT produced therapeutic effects, suspected in the form of immediate termination of apoptotic rate, possibly through the improvement of pro-and antiapoptotic protein ratio, thereby increasing the expression of GDF-9. In addition, this

extragonadal BMT releases progenitor germ cells into the circulation and then it was "homing" to the ovaries and engrafted within the follicle. Another possible mechanism may be the transformation to improve the microenvironment in ovarian follicles (Johnson, 2007).

This study showed the data on the difference of Kit-Ligand expression in the three groups above. Kit-Ligand expression in cisplatin group (12.27 ± 2.88) was lower than that in control group (20.22 ± 2.14), which means that there has been damage or apoptosis in granulosa cell due to intraperitoneal cisplatin administration, resulting in low expression of Kit-Ligand. In group 3 that received BMT, Kit-Ligand expression (20.26 ± 1.14) was higher than cisplatin group (12.27 ± 2.88). These results suggest that BMT provides the same therapeutic effect on granulosa cells and oocytes in the form of improved ratio of pro- and anti-apoptotic proteins. In addition, as in the oocyte, BMT progenitor germ cell was "homing" to the ovaries and engrafted in the follicle, thus improving the expression of Kit-Ligand.

Further results in this study showed that the number of follicular development in the cisplatin group in the form of primordial (4.31 ± 1.19), primary (3.81 ± 1.22), secondary (2.87 ± 0.95) and graafian (0.37 ± 0.69) follicles were lower than the control group, which was (6.12 ± 1.20), (4.93 ± 1.61), (4.25 ± 0.77) and (5.81 ± 1.37), respectively. Follicle is the functional unit of the female reproductive organs, consisting of 3 cells: granulosa, theca cell and oocyte. Folliculogenesis is a growth and

development process involving endocrine and molecular interactions between the 3 cells, particularly between oocyte and granulosa cells with the final result mature and fertilizable oocyte (Rajkovic et al., 2006). These results confirm that the administration of cisplatin will damage oocyte and granulosa cells and interfere with secreted growth factor. The subsequent result is the disrupted interaction of oocyte and granulosa cells and in folliculogenesis process, resulting in the reduction of a significant number of follicular development in the group receiving cisplatin. Therefore, the number of follicular development in BMT group was higher than cisplatin group. This situation means that the therapeutic effects of BMT successfully repair damaged oocyte

and granulosa cells and their growth factor production. The final result is folliculogenesis process improvement, as reflected by the higher number of follicular development in BMT group. The conclusion of this study is that in cisplatin-induced ovarian failure in rat, bone marrow transplantation may improve oocyte-granulosa cell interaction and follicular development. Further study is required to confirmation that result, with some improvement of methodology such as: using GFP to trace the BMT in cells, add sham group (PBS and BMT) as a control, perform ELISA to confirm enhanced GDF-9 secretion and exploration the optimal dose of BMT.

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