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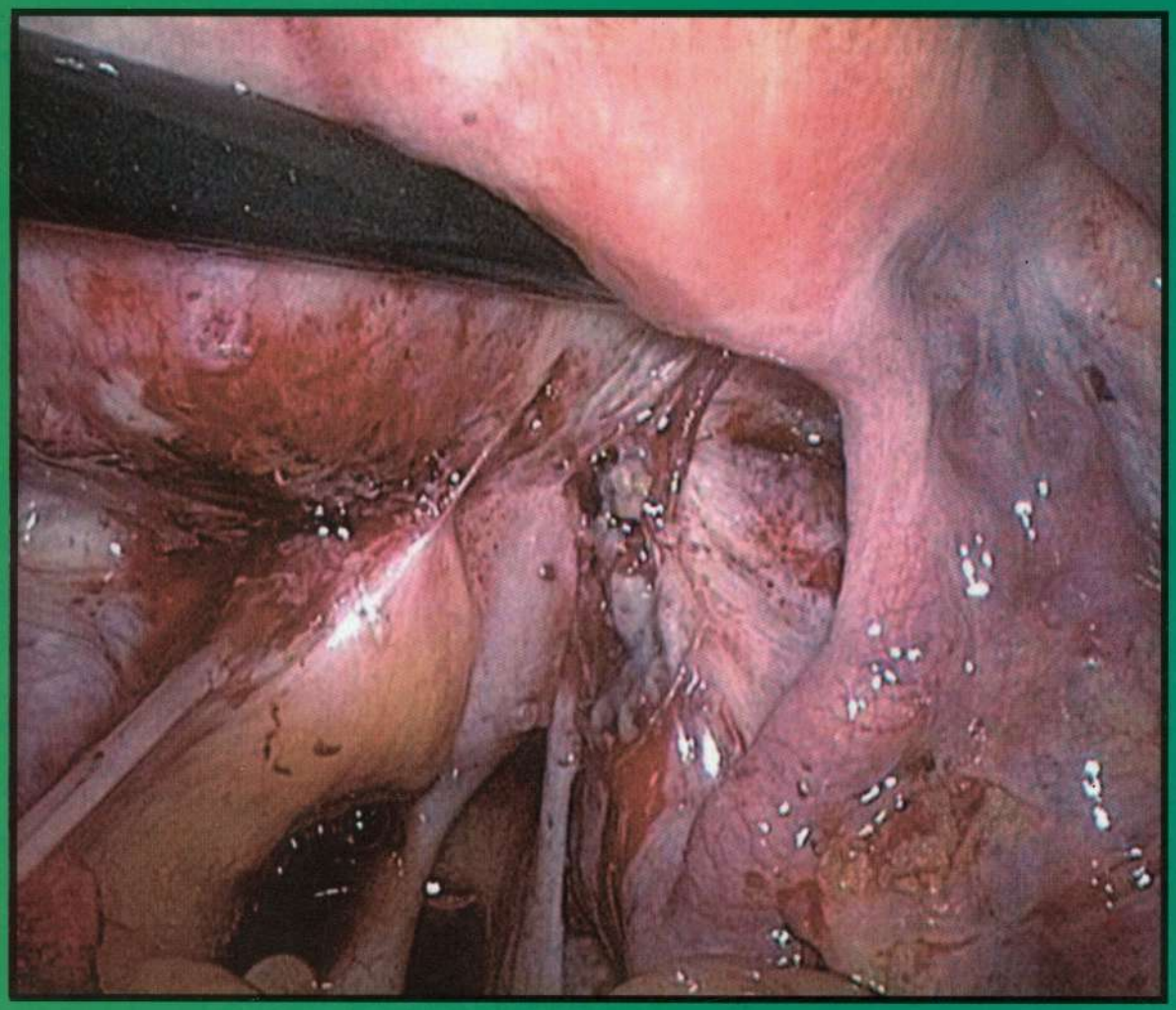

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Expression of Kit Ligand and Amount of Follicles as Features of Folliculogenesis Disorder on Rat (*Rattus novergicus*) Strain Wistar with Cisplatin

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ABSTRAK

*Kit Ligan (KL), salah satu faktor pertumbuhan pertama dalam folikel ovarium, memainkan peran kunci dalam oogenesis mamalia dan folliculogenesis. KL protein dan ekspresi mRNA terdeteksi dalam sel granulosa dari folikel pada semua tahap perkembangan. Cisplatin adalah salah satu senyawa platinium generasi pertama dan umumnya digunakan untuk pengobatan ovarium, testis payudara, dan kanker kandung kemih.Tujuan dari penelitian ini adalah untuk membuktikan ekspresi yang lebih rendah dari ligan kit dan jumlah folikel, dan hubungan antara ekspresi ligan kit dan jumlah folikel, pada tikus (*Rattus novergicus*) strain wistar dengan cisplatin. Penelitian ini dilakukan di Laboratorium Hewan Coba dan Patologi Veteriner, Fakultas Kedokteran Hewan, Universitas Airlangga, Surabaya, Mei-Juli 2011. Penelitian ini dilakukan oleh randomized post test only control group design. Sampel penelitian ini adalah tikus (Rattus novergicus) galur wistar yang sesuai dengan kriteria inklusi. Pada periode diestrus, kelompok kontrol disuntik oleh IP NaCl 0,9%, dan kelompok cisplatin disuntikkan dengan cisplatin IP 5 mg/kg w. Tikus dibantai pada hari ke-7 periode estrus pada kelompok kontrol, dan pada periode diestrus pada kelompok cisplatin. Ooforektomi dilakukan, dilanjutkan dengan pewarnaan immuno-histokimia dan hematoxylin eosin untuk pemeriksaan ekspresi ligan kit dan jumlah folikel. Tidak didapatkan hubungan antara ekspresi ligan kit dan jumlah folikel primordial, primer, sekunder, dan tersier ($p = 0,945$, $p = 0,180$, $p = 0.590$, $p = 0.753$) pada tikus dengan cisplatin. Sebagai kesimpulan, pada tikus dengan cisplatin, ekspresi ligan kit, jumlah folikel primordial, primer, sekunder, dan tersier rendah. **(MOG 2011;19:102-108)***

Kata kunci: *ekspresi ligan kit, jumlah folikel, cisplatin, folikulogenesis, tikus*

ABSTRACT

*Kit Ligand (KL), one of the first growth factors in ovarian follicle, plays a key role in mammalian oogenesis and folliculogenesis. KL protein and mRNA expression is detected in the granulosa cells of follicles at all stages of development. Cisplatin is one of the first generation platinium compounds and is commonly used for treatment of ovarian, breast, testicular, and bladder cancers.The objective of this study was to prove the lower expression of kit ligand and amount of follicles, and the association between the expression of kit ligand and the amount of follicles, on rat (*Rattus novergicus*) strain wistar with cisplatin. This study was conducted at Laboratory of Experimental Animal and Veterinary Patology, Faculty of Veterinary, Airlangga University, Surabaya, May-July 2011. The research was performed by randomized post test only control group design. The research samples were Rat (Rattus novergicus) strain wistar which was admitted into inclusion criteria. At the diestrus period, the control group was injected by NaCl 0.9% IP, and the cisplatin group was injected by cisplatin 5 mg/kg w IP. The rat were slaughtered at the 7th day of estrus period on the control group, and at the diestrus period on the cisplatin group. Oophorectomy was performed, continued with immuno-histochemistry staining and hematoxylin eosin for examination on expression of kit ligand and amount of follicles. We achieved no association among the expression of kit ligand and the amounts of primordial, primary, secondary, and tertiary follicles ($p=0.945$, $p=0.180$, $p=0.590$, $p=0.753$) on the rat with cisplatin. In conclusion, In rat with cisplatin, the expresssion of kit ligand, the amount of primordial, primary, secondary, and tertiary follicles are low. **(MOG 2011;19:102-108)***

Keywords: *expression of kit ligand, amount of follicles, cisplatin, folliculogenesis, rat*

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INTRODUCTION

Over the past three decades, there has been a remarkable improvement in the survival rates due to progress in cancer treatment.[1] In the past, the primary goal of cancer therapy—survival—tended to overshadow survivorship considerations. However, with recent advances in oncology, survival rates are increasing, and therefore, issues affecting long-term cancer survivors become more important and more widely recognized.[2] As a consequence of the increase in the number of patients surviving cancer, greater attention has been focused on the delayed effects of cancer treatments on the quality of future life of the survivor.[1] An unfortunate and devastating consequence of chemotherapy is ovarian damage; leading to diminished fertility potential and

lower post treatment birth rates in female cancer survivors. Specifically, malignancy on women 1179 and 1261 people. While data on Pharmacy Installation of dr Soetomo State Hospital showed that the amount of female patients with malignancy and receive cisplatin chemotherapy at 2009 and 2010 consecutively are 300 and 457 patients.

Young patients treated with chemotherapy or radiotherapy may suffer from gonadal damage and permanent ovarian failure. Most studies on the deleterious effects of chemotherapy on human ovaries have focused on indirect parameters of ovarian failure, such as menstrual history, hormone levels and subsequent pregnancies. Ovarian failure after treatment was found to be related to the age of the patient, and treatment protocols. Histological studies of human ovaries demonstrated that the end result of chemo-therapy was ovarian atrophy and anticancer drugs diminish the primordial follicle pool, cause ovarian atrophy, and harm the ovarian blood vasculature.3 The number of childhood cancer survivors in the United States was 270,000 in 2003, and the prevalence of cancer survivors among young adults (15 to 45 y of age) in the United States will be 1 in 250 people in 2010.4 Cancer affects over 11,000 global loss of primordial follicles.[6] The study uses experimental animal such as rat (*Rattus novergicus*) strain wistar for replacing human for more invasive research which has been inhibited by ethic on the execution. Folliculogenesis is the process by which the female germ cell develops within the somatic cells of the ovary and matures patients per year in the UK in the 15–40 into a fertilizable egg.[7]

The follicular years age group, comprising 4% of patients with cancer.[5] Data of year 2009 and 2010 in Department of Anatomic Pathology Faculty of Medicine Airlangga University/dr Soetomo State Hospital Surabaya shows that amount of women with age 15-35 years old who suffered from malignancy consecutively are 178 and 169, from total development starts from the smallest primordial follicles recruited into the growth pool through primary, preantral, and antral stages to the largest Graafian or preovulatory follicles (POFs) that ovulate in response to the luteinizing hormone (LH) surge. After ovulation, the remaining granulosa cells and theca cells within the POFs differentiate into luteal cells to form the corpus luteum (CL).[8]

Folliculogenesis can be divided into two phases. The first phase, termed the preantral or gonadotropin-independent phase, is characterized by the growth and differentiation of the oocyte. The second, termed the antral or gonadotropin-dependent phase, is characterized by the tremendous increase of the size of the follicle itself (up to approximately 25-30 mm). The preantral phase is controlled mainly by locally produced growth factors through autocrine/paracrine mechanisms. The second phase is regulated by FSH and LH as well as by growth factors. There is great interest in the growth factors because they can stimulate cell proliferation and modulate gonadotropin action.[8]

During the last decade, the role of growth factors in ovarian folliculogenesis has been extensively studied in several species, including rodents, domestic animals, and humans. In particular, Kit Ligand (KL), which was one of the first growth factors identified in the ovarian follicle, plays a key role in mammalian oogenesis and folliculogenesis. Since its identification in 1990, in vivo and in vitro studies have shown that the functions of this system in the ovary include the establishment of primordial germ cells (PGCs), activation of primordial follicles, oocyte survival and growth, proliferation of granulosa cells, recruitment of theca cells, and maintenance of meiotic competence.[9]

In post-natal mouse ovaries, KL protein and mRNA expression is detected in the granulosa cells of follicles at all stages of development, though its expression is very low in primordial and primary follicles, and in the cumulus cells of antral follicles. While only limited primordial follicle granulosa cell staining is detected in mice, more than 90% of primordial follicles in sheep consist of at least one granulosa cell expressing KL protein. Moreover, KL protein is detected in the oocytes of resting and growing murine follicles, presumably as a consequence of receptor-mediated endocytosis.[10]

Cisplatin is one of the first generation platinium compounds and is commonly used for treatment of ovarian, breast, testicular, and bladder cancers. Cisplatin exerts its cytotoxic effects by covalently binding to DNA with preferential binding to N-7 positions of guanine and adenine, producing intrastrand and interstrand cross-links. Cisplatin also binds to nuclear and cytoplasmic proteins, which may cause toxic effect.[11]

MATERIAL AND METHODS

The study was laboratory experimental research on experimental rats with randomized post test only control group design. The research subjects were divided randomly into two groups, one group as the control and the other one received cisplatin and the variable measurement were performed at the end of the research. The research was performed at May-July 2011, in Experimental Animal Laboratory and Veterinary

Pathology Laboratory Faculty of Vetrinary Medicine Airlangga University Surabaya.

The research sample was rat (*Rattus novergicus*) strain female wistar, three months old, weigh 150-200 grams. The research samples were divided into two groups, the P0 group receives NaCl 0.9% injection intraperitoneal (IP) at diestrus period and the PI group receives cisplatin injection 5 mg/kgw IP at diestrus period, then on both groups were performed ovarian surgery by the 7th day at estrus period. The amount of samples were 16/groups based on Federer-Federer samples, so the whole total of sample is 32 rats.

Inclusion criterias included female rat (Rattus novevrrgicus) strain wistar, three months old, weigh 150-200 grams, virgin (no history of copulation), healthy, signed with tender feather, shiny eyes, not lame, no presence of scar. Exclusion criterias included history of being used as other research's experimental animal, sick rat drop-out criteria of experiment, dead, or trapped by the cage after getting treatment.

Independent variable was cisplatin. Dependent variables were primordial follicles, primary follicles, secondary folllicles, tertiary follicles, and expression of kit ligand. While control variables were type of experimental animals, sex of experimental animals, age of experimental animals, physical health, maintenance and care of experimental animals, and the making of histological preparation.

All experimental animals used were taken from Utomo Farm of Batu City. The experimental animal that we used were female rat (*Rattus novergicus*) strain wistar aged about three months with weigh 150-200 grams, selected based on inclusion criteria and not include to exclusion criteria. Adaptation of experimental animals lasted for a week before in a clean, enough of air and light cage, with enough food and drink and homogen. Those rats were divided into two groups which each consist of 16 rats randomly. At diestrus period, the P0 group received treatment by NaCl 0.9% injection IP, the P1 received treatment by cisplatin injection 5 ml/kgw IP.

On the both groups of rat at estrus period at the seventh day, performed surgery and oophorectomy. Then the ovaries were given codes. The ovaries were sent to Laboratory of Veterinary Pathology for creating histological preparation and performed hematoxilin-eosin staining to count amounts of primordial, primary, secondary, and tertiary follicles seen microscopically. Immunohistochemistry staining were performed to see the expression of kit ligand. After the preparation had been read, they were performed code opening, and the data of the study were recorded in forms of data recording. Data analysis used software self propelled semi submersible (SPSS).

We performed examination on the expression of KL and the amount of follicles on the control and the cisplatin groups. The rat's weight was tested by Levine's Test to see the sample homogenity. Data normality test used Kolmogorov Smirnov test for testing the data normality of the expression of kit ligand and the amount of follicles. The data of the study distributed normally, so the comparation test were performed with T- paired test. Correlation test was performed by Pearson Test. We used significancy level in the study about 0.05. Ethical properness was achieved from Animal Care and Use Committee Faculty of Veterinary Medicine Airlangga University Surabaya.

RESULTS AND DISCUSSION

The weigh of rat that we used in the cisplatin and the control groups, $p = 0.0001$. Research ranged around 150-175 grams. On the cisplatin group, we achieved the mean of the pre-treatment weigh 159.06 ± 7.12 grams and the mean of the post-treatment weigh 165.63 ± 7.72 grams. While on the control group we achieved the mean of early weigh was 161.56 ± 7.89 grams and the mean of end weigh was 168.75 ± 6.95 grams. Then we performed homogenity test on data of the early and the end weigh of the both groups by Levine's Test. The result was, the early weigh ($p = 0.396$) and the end weigh ($p = 0.69$) were homogenous. The study intended to explain the folliculogenesis impairment on rats which got cisplatin. The analyzed variables were expression of kit ligand and amount of folllicles.

Then we performed test of data normality on the research variables. The test of normality data was performed by Kolmogorov-Smirnov test with significancy level 0.05.The results of normality test Kolmogorov-Smirnov showed that the amount of the expression of kit ligand ($p = 0.922$), the amount of primordial ($p = 0.381$), primary ($p = 0.386$), secondary ($p = 0.1$) and tertiary follicles ($p = 0.06$) distributes normal with $p > 0.05$. By the normal distribution result, the comparation test was performed by using T-paired test.

On the study, we achieved the ampunt of the expression of kit ligand on cisplatin group was 12.19 ± 2.95 and on the control group was 20.17 ± 2.10. After we performed comparation by T-paired test, we got significant difference between the cisplatin and the control groups, $p = 0.0001$. We counted the amount of follicles and found that on cisplatin group, the amount of primordial

follicles was 4.31 ±1.19, the primary follicles was 3.81 ± 1.22, the secondary follicles was 2.88 ± 0.96 and the tertiary follicles was 1.38 ± 0.50. While on the control group, the amount of primordial follicles was 6.13 ± 1.2, the primary follicles was 4.94 ± 1.61, the secondary follicles was 4.25 ± 0.78 and the tertiary follicles was 3.69 ± 1.19. Then we performed comparation test by T-paired test, we achieved significant difference between cisplatin and control groups on all follicles, with significancies of primordial follicles p = 0.0001, primary follicles p = 0.034, secondary folicles p = 0.0001, and tertiary follicles p = 0.0001.

To find out the association between the expression of kit ligand and the amount of follicles on cisplatin group, we performed correlation test. The correlation on this study used the Pearson correlation test because the test of data normality resulted to normal distributed data. Correlation test on cisplatin group resulted that, the association between expression of kit ligand and primordial follicles was p = 0.945, expression of kit ligand and primary follicles was p = 0.18, expression of kit ligand and secondary follicles was p = 0.59, expression of kit ligand and tertiary follicles was p = 0.753. So, the result of correlation test was not significant because p > 0.05.

DISCUSSION

The adult mammalian ovary is a complex organ composed of various cell types including oocytes, granulosa, theca, stroma and surface epithelial cells. These cell types are further divided into various subtypes. For example, the granulosa cells can be further differentiated into mural, cumulus, corona radiata or luteal cells, while theca cells develop into internal, external and luteal cells. The coordinated control of proliferation, differentiation and apoptosis of these cell types forms the underlying basis for menstrual or estrous cycles in mammals. The mechanism by which each cell type obtains its state of proliferation and/or differentiation is the subject of intense study and it has been shown that, as well as endocrine compounds, locally produced factors can regulate or modulate these developmental processes.[12]

Kit ligand (KL), encoded by the Steel (Sl) gene, is a locally produced factor that is thought to have many roles in ovarian function. KL mRNA expression in follicles is, however, localized to granulosa cells in all species studied so far and can be expressed as either a membrane-bound or a soluble protein, depending on how the mRNA is spliced. Both transcripts, when translated, yield membrane-associated products, but KL-1 is efficiently cleaved and released as a soluble product due to a proteolytic cleavage site encoded by an 84- base pair exon. The other form, KL-2, lacks this cleavage site and therefore remains membrane-bound. The membrane-bound KL is the more potent of the twoformswith regard to its ability to induce the proliferation of primordial germ cells. Both membrane-bound and soluble forms of KL are present in the mouse ovary.[12]

The process by which a follicles matures from the primordial to the preovulatory stage, with many steps in- between is referred to as folliculogenesis. This development involves two major processes, i.e., recruitment of the follicle into the growing pool and the proliferation and differentiation of the granulosa and theca cells. The first of these processes is regulated by paracrine and autocrine signals produced in the ovary itself; while the second is controlled both by this internal signalling and by endocrine signals from outside the ovary.[13]

The follicle begins as an oocyte surrounded by a single layer of GCs enclosed in turn, by a basement membrane. During development, the proliferating GCs provide nutrients and various molecular signals to the oocyte, which increases in size. Re-organization of the follicle and the differentiation and proliferation of the GCs results in the formation of an antrum prior to ovulation. In these ways, the follicles supports the oocyte, both chemically and physically. During this development process the follicle migrates from the cortex to the medulla and then back again as ovulation approaches.[13] Cisplatin or cis-diamminedichloro platinum is an inorganic compound that is widely prescribed for a variety of tumours (germ-cell tumours, advanced bladder carcinoma, adrenal cortex carcinoma, breast cancer, head and neck carcinoma, lung carcinoma).[14]

The rat weigh of the control and cisplatin groups were performed homogenity test by Levene's test, and we found that there was no significant differrence between the control and cisplatin group before and after treatment. So, the weigh of the rats were homogenous. On the study, before treatment, all rats were performed vaginal smear to equal the menstruation cycle. Rats on diestrus period were given treatment. The control group was injected by NaCl 0.9% IP and the cisplatin group were injected by cisplatin 5 mg/kgw IP. At the seventh day after treatment, before the rats were slaughtered, they were performed vaginal smear. The result of vaginal smear on the control rats showed estrus period, while on the cisplatin group showed diestrus period. The diestrus feature was suitable to the studies of Rivera et al and Schauwecker et al, where the ovarian failure was determined when the vaginal cytology showed > 15 days of persistent diestrus.[15,16]

On this study, the expression of KL on cisplatin group was lower than the control group. After we performed the comparation test by T-paired test, we found the significant difference. It supported the following studies. In order to visualize and study the direct mechanism of chemotherapy-induced ovarian damage and primordial follicles injury, healthy human cortical ovarian slices were exposed in vitro to therapeutic doses of cisplatin. Histology and immunohistochemical staining showed that chemoterapy induced pregranulosa cell swelling, marked pregranulosa cell nuclear swelling and primordial follicles architecture disruption with disappearance of the lumen and its oocyte. Positive apoptotic staining was obtained in the pregranulosa cells exposed to chemotherapy but not in controls. Therefore, in the human ovary cisplatin acts primarily on the pregranulosa cells inducing apoptotic changes, and the cells exhibit marked swelling. Familiari et al examined ovarian follicles by electron microscopy following expossure to chemotherapy. Results have shown that follicular cells were enlarged containing cytoplasmic elements and the nuclei within the pregranulosa cells were also enlarged. Also the primordial follicles population was often surrounded by an abnormally thick basal lamina.[17]

In the ovary, c-kit is expressed in oocytes, and KL is produced by granulosa cells and is thought to function in oocyte growth in a paracrine manner. Expression of c-kit is found in oocytes as early as in the primordial follicles of newborn mice and the sheep fetus. In the mouse, inhibition of the interaction between KL and c-kit by the specific antibody prevents transition from primordial follicles to primary follicles without blocking the formation of primordial follicles. Administration of anti c-kit antibody (ACK2) to mice has even stopped the proliferation of granulosa cells.

Therefore, KL/c-kit interaction appear to be essential for the growth initiation of mouse oocytes. In addition, Packer et al have shown with in vitro cultures that KL (10-100 ng/ml) promotes the growth of oocytes collected from 8-days-old mice. Parrot and skinner have used rat ovaries and shown that KL (100 ng/ml) induces a significant development of primordial follicles. The promotional effect of antrum formation by KL (20 or 50 ng/ml) has also been reported in cultured mouse preantral follicles. However, these promotions by KL have not been confirmed in other species.[18]

The study result showed that the amount of primordial, primary, secondary, and tertiary follicles on cisplatin group were lower than the control group. Comparison test result by T-paired test showed that the amount of primordial, primary, secondary, and tertiary follicles on cisplatin group were different significantly than the control group. It supported the following studies.

Chemotherapy has been suggested to induce damage to primordial follicles by inducing apoptosis. Electron microscopy has shown that within hours of chemotherapy exposure, primordial follicles become surrounded by abnormally thick basal lamina. A substantial body of evidence has documented in vitro evidence of primordial follicle apoptosis and chemotherapy treatment in vitro has also been shown to cause primordial follicle architecture disruption and pregranulosa cell swelling. Preliminary in vivo studies using human ovarian xenografts in SCID mice have also shown indications of primordial follicle apoptosis. More research is needed to verify that primordial follicle apoptosis does indeed occur in true in vivo conditions, and whether the oocyte or surrounding granulosa cells are the primary target. Alternatively, other mechanisms of damage, such as cortical fibrosis and follicular ‘‘burn-out’’ have been suggested to explain the variable loss of follicular reserves.[3]

It is clear that chemotherapy results in ovarian cortical fibrosis and blood vessel damage. In a study conducted on human ovarian tissue exposed to combination chemotherapy in vivo, hyalinization of cortical blood vessels, neovascularization, and cortical fibrosis were observed. These modes of injury result in local ischemia, thereby affecting the growth and survival of primordial follicles. Triangular areas of fibrosis have been observed to coincide with a depletion of primordial follicles, indicating that blood vessel damage results in primordial follicle injury. This may also impair the processes of new vessel formation that are critical for normal follicle growth within the territory of the damaged vasculature. If apoptosis had initially led to a diminished need for blood vasculature, then a uniform pattern of primordial follicle loss would be expected; this however, is not the observed trend.[3]

The correlation test between KL expression and amount of follicles on the research was not significant, showed that there was no association among the amount of KL expression and the amounts of primordial, primary, secondary, and tertiary follicles on cisplatin group. There were some things that we thought as the causes of the abscence of the association between KL expression and the amount of follicles. The statistic accounting which stated that there was no significant association between the amount of the expression of kit ligand and the amount of the follicles, showed that there was still other factors which also played role on folliculogenesis process. As we know, that folliculogenesis is the process by which the female germ cell develops within

the somatic cells of the ovary and matures into a fertilizable egg.[7]

Ovarian follicle is the functional unit of woman's reproduction, consists of oocyte, granulose cell, and theca cell. Folliculogenesis involved corporation among those three cells and included lots of process such as: proliferation of granulose cells, oocyte meiosis, steroidogenesis of theca cells, expansion of cummulus. So, we need to perform exploration to those factors to explain the mechanism of the impairment on the folliculogenesis.

CONCLUSION

In rat with cisplatin, the expresssion of kit ligand, the amount of primordial, primary, secondary, and tertiary follicles are low.

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