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Effects of Platelet-Rich Plasma on Ovary Function in Rats with Cyclophosphamide Induced Ovarian Damage

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Abstract: Background: The term premature ovarian failure is defined as a persistent decrease in ovarian function in women. Platelet-rich plasma contains growth factors. Our study aimed to test the effectiveness of platelet-rich plasma that was tried in patients who did not benefit from routine infertility treatments on experimental animals. **Material and Methods:** In the study, 40 female rats were used, and to obtain platelet-rich plasma, eight male rats were used. The animals used in the study were randomly divided into four groups, with ten animals in each group prospectively. At the end of the 21st day, rats in all groups were euthanized by taking intracardiac blood. Following anti-mullerian hormone measurement, histological analysis, follicle count, and real-time-polymerase chain reaction analyzes were made. **Results**: In our study, the highest anti-mullerian hormone concentration was measured only in the group given platelet-rich plasma, and the lowest value was measured in the group with ovarian damage. The results of Bcl-2 mRNA expression analysis by real-time-polymerase chain reaction also showed that the lowest expression occurred in the group with ovarian damage. The correlation analysis results showed a statistically significant, moderate negative correlation between the ovarian damage group and the ovarian damage + Platelet-rich plasma group. **Conclusions**: As a result, it is evaluated that platelet-rich plasma can be used as a preventative in women who are treated with cyclophosphamide and similar agents and subsequently develop ovarian side effects that are very difficult to treat.

Keywords: Platelet-Rich Plasma, Cyclophosphamide, Anti-Mullerian Hormone, Bcl-2, Real-Time- Polymerase Chain Reaction.

INTRODUCTION

The term premature ovarian failure (POF) is defined as a persistent decrease in ovarian function in women, often leading to premature menopause (Jankowska, K. *et al*., 2017). POF is detected with a frequency of 1% under the age of 40 and 0.1% under 30 in women. The etiology is unknown in most cases. The incidence of POF has increased in recent years. Exposure to some iatrogenic agents may cause POF proportionally with duration and dose [Tsiligiannis, S. *et al*., 2019].

Cyclophosphamide (Cy) is a chemotherapeutic agent frequently used to treat various cancers, especially hepatic and renal cancers. Despite its cytotoxic side effects, it is effective alone or in combination therapy. Today, chemotherapy-induced gonadotoxicity is a fundamental cause of POF due to women's increasing incidence of cancer (Sung, H. *et al*., 2021). Every year, thousands of women in the reproductive period are exposed to cytotoxic forms of chemotherapy for cancer treatment. One of the most crucial side effects of these treatment forms is the development of POF in these patients due to their gonadotoxic effects on the ovarian tissue. Cy has a particularly detrimental effect on the ovaries. The toxic mechanism of action is related to the disruption of DNA synthesis [Luan, Y. *et al*., 2019; Kim, S. *et al*., 2021].

Platelet-rich plasma (PRP) is autologous plasma with platelet content at least four times the average platelet concentration. PRP contains growth factors related to wound healing and tissue repair and plasma proteins that provide hemostasis and adhesion. Although there are studies investigating the effect of PRP on the atrophic ovary, which has previously been found to have positive effects on regeneration and angiogenesis in many tissues, its benefit has not been fully elucidated [Budak, Ö. *et al*., 2020- Atkinson, L. *et al*., 2021].

Anti-Müllerian Hormone (AMH) is secreted by the granulosa cells of small follicles in the ovary in women. Serum values increase rapidly with the onset of puberty. Measurement of serum AMH levels is an ideal tool for assessing ovarian follicular reserve. In many studies, studies are accepted that AMH is a valuable marker in showing the number of growing follicles [Moolhuijsen, L.M. *et al*., 2020].

Bcl-2 gene family proteins function as regulators of apoptosis, regulating the process by which mitochondria contribute to cell death. In addition, it plays a critical role in processes such as neuronal activity, autophagy, calcium processing, and mitochondrial dynamics. In studies with mice, it has been shown that deficiency or excess of Bcl-2 proteins causes changes in the number of follicles (Hardwick, J.M. *et al*., 2013).

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Our study aimed to examine the efficacy of PRP in experimental animals with premature ovarian failure by using different analysis methods.

MATERIALS AND METHODS

Experimental Animals

In the study, 40 female and eight male rats were used to obtain PRP. Female rats are 8-10 weeks old, adult Wistar breed with 40 regular cycles, weighing 120±40 grams. Male rats are 8-10 weeks old, adult Wistar breed, weighing 200±50 grams. The animals were kept in a 12-hour light, 12-hour dark photo-period, and a constant temperature room of 21-23 °C and were fed standard pellet feed and city water. Experimental animals were randomly divided into four groups, with ten animals in each group. The control group was given physiological saline; the other groups were given Cy, Cy + PRP, and only PRP. The relevant Ethics Committee approved the protocol regarding the procedures performed on animals. All work and procedures were carried out following the Guide for the Care and Use of Laboratory Animals (Clark, J.D. *et al*., 1997).

PRP Obtainment

Eight male Wistar rats were euthanized by intracardiac route, and blood was drawn for obtaining PRP. The collected blood was placed in tubes containing 3.2% sodium citrate (blood/citrate ratio: 9/1) and centrifuged at 400 g for 10 minutes. The supernatant and the part with platelet-rich plasma were taken into a separate tube. This tube was centrifuged at 800 g for 10 minutes, and the precipitated part was collected. The resulting PRP was prepared to be four times the platelet concentration in normal blood per microliter. In our research, freshly obtained PRP was used for each analysis

Study Design and Collection of Samples

The animals used in the study were randomly divided into four groups, with ten animals in each group prospectively (Dehghani, F. *et al*., 2018).

Intraperitoneal saline %0.9 was administered to group 1 (Control group) on days 1-8-15.
For group 2, Cy 75 mg/kg/single dose was administered intraperitoneally on day 1, and the serum was administered physiologically at %0.9 1 mL/kg on days 8-15.
For group 3 (Cy + PRP group), Cy 75 mg/kg/single dose was administered intraperitoneally on day 1, and PRP 200 microliters were administered on days 1-8-15.
For group 4 (PRP), PRP 200 microliters were administered intraperitoneally on days 1-8-15.

The study lasted 21 days. A single dose of 75 mg/mL Cy caused detectable ovarian damage at the end of 21 days. At the end of the 21st day, rats in all groups were euthanized by taking intracardiac blood under intramuscular ketamine (45mg/kg) + xylazine (5mg/kg) anesthesia. After euthanasia, ovarian tissues were quickly removed, fixed with appropriate fixatives, and embedded in paraffin blocks. Hematoxylin-eosin dyes were applied to the sections taken from paraffin blocks with a thickness of 5-6 mm and examined under the research microscope. The histopathological changes were recorded according to the groups, and the follicles were separated and counted according to the groups. At the beginning of the experiment and the end of the experiment, AMH hormone measurement was made in the serum of the rats. The blood taken for biochemical analysis was centrifuged at 4500 rpm, serum was extracted, and AMH hormone was measured. In addition, Bcl-2 mRNA expression analysis was performed by RT-PCR from ovarian tissue.

AMH Measurement

AMH was measured with a Rat Mullerian Inhibiting Substance/Anti-Mullerian Hormone ELISA measurement kit from Bioassay Technology Laboratory (Bioassay Technology Laboratory, Shanghai, China). The reading process was performed on a Chromate 4300 (Awareness Technology, Inc. Martin Hwy. Palm City, USA) ELISA reader device. Results are given in mg/L.

Histological Analysis

The researchers carried out a histopathological examination. The researcher was unaware of the groups during the study. The removed ovaries were kept in 10% formalin for 72 hours, then cleaned with alcohol and xylene, increasing from 70% to 100%. The samples were coated with paraffin at 60°C after cleaning. Sections of 5 µm thickness were taken from the paraffin blocks for analysis. Sections were stained with hematoxylin & eosin and examined under a photomicroscope (Nikon Eclipse i5, Tokyo, Japan).

Follicle Count

Multiple sections were taken from each ovary to determine the number of follicles. Those with oocyte nuclei from the follicles were included in the analysis and classified into five groups. Primordial, primary, secondary, antral, and atretic follicles form five groups.

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Real Time-PCR
Primarily, ovarian tissues were homogenized for analysis. For this purpose, a tissue homogenizer (Next Advance, USA) was used. According to the manufacturer's instructions, RNA isolation was performed using the PureLink RNA Mini Kit (Invitrogen, USA).

Accordingly:
The appropriate volume of lysis buffer containing 2-mercaptoethanol was added to the sample. At room temperature, the lysate below was placed in the collection tube and centrifuged for two minutes at 12,000 g.

1.5 volumes of 100% ethanol and tissue lysate were added to an appropriately sized RNase-free tube and vortexed.

700 µL of the sample was transferred to the cartridge (Spin Cartridge) and transferred to the collection tube, centrifuged at 12,000 g for 15 seconds at room temperature, and the liquid part was discarded. 350 µL wash buffer 1 was added to the cartridge (Spin Cartridge), centrifuged for 15 seconds at 12,000 g at room temperature.

80 µL of DNase mixture was added to the surface of the cartridge membrane and incubated for 15 minutes at room temperature.

Once more, wash buffer 1 was added and centrifuged. 500 µL of washing buffer 2 was added to the cartridge with ethanol, centrifuged at room temperature, and the liquid part was discarded.

The cartridge was placed in a recovery tube (Recovery Tube), added RNase-free water, and incubated for one minute at room temperature. Centrifuged for 2 minutes at 12,000 x g, the liquid portion was discarded.

The quantity and quality of purified total RNA were determined with the Quant-iT™ RiboGreen™ RNA Test Kit using a fluorescent microplate reader (UV absorbance 260 nm).

The obtained RNA was stored at -80oC.

cDNA synthesis was done in a palm cycler device.

While interpreting our results, the concentration value of our target genes was proportioned to the concentration value of the reference (housekeeping) gene, and the variation of the results obtained compared to the control group were examined. In our study, the beta-actin gene was used for this purpose.

$2^{-\Delta\Delta Ct}$ Calculation
Accuracy and reliability in PCR analyses are related to the efficiency of PCR. For this purpose, the mRNA expression level of the target gene (Bcl-2) is encountered with the reference gene (Beta Actin), the $2^{-\Delta\Delta Ct}$ method is widely used. In our study, the Bcl-2 expression values of the samples were transformed by the $2^{-\Delta\Delta Ct}$ method. Statistical analyzes were made on the values obtained.

Statistical Analyses
The Kruskal Wallis test was used to examine the significant difference between the groups. When a statistically significant difference was detected between the groups, the Mann-Whitney U test was used to determine which groups caused the difference. In the research, Spearman Correlation analysis was performed to determine the correlation between the groups.

Ethics
The necessary ethics committee permissions for our study were obtained from the relevant committees.

RESULTS

Average serum AMH concentrations obtained in our study are shown in Table 1. Accordingly, the highest AMH concentration was measured only in the group given PRP without ovarian damage. This group is followed by the control and ovarian damage + PRP groups. According to the statistical analysis, there was a significant difference between the groups ($p<0.01$). The results of the analysis showed that the difference was due to the fact that the AMH values of the group with ovarian damage were considerably lower than all other groups.

In our study, Bcl-2 mRNA expression analysis was performed by RT-PCR in ovarian tissue. The analysis results were transformed with the 2-ΔΔCt method, and statistical analyses were made over the converted values. The results were similar to the AMH analyses. A significant difference was found between the groups ($p<0.01$), and as a result of further statistical analysis, it was determined that the difference was due to the lower 2-ΔΔCt values of the group with ovarian damage compared to all other groups (Table 1).

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Table 1: Anti Mullerian and 2 Base Delta Ct (Bcl-2) Analysis by Groups*

Groups		n	Mean Rank	SD	x^2*	p	Significant Difference (Mann Whitney U Test)
Anti Mullerian Hormon	Control (1)	11	26.2727	3	24.44	**0.00**	**2<1,3,4** **3>4**
	Ovarian damage (2)	11	8.90909				
	PRP (3)	11	35.1364				
	Ovarian damage + PRP (4)	11	19.6818				
$2^{-\Delta\Delta Ct}$ (Bcl-2)	Control (1)	11	27.6364	3	19.96	**0.00**	**2<1,3,4**
	Ovarian damage (2)	11	8.27273				
	PRP (3)	11	30.8636				
	Ovarian damage + PRP (4)	11	23.2273				

*Kruskal Wallis Test

According to the results of the correlation analysis, a statistically significant, moderate negative correlation was found between the group with ovarian damage and the group with ovarian damage who underwent PRP ($r=-0.622$; $p<0.05$) (Table 2).

Primordial, primary, secondary, antral, and atretic follicle counts were made for all groups. There was no significant difference in the number of primordial, primary, secondary, antral, and atretic follicles between the groups. Primordial, primary, secondary, and antral follicle counts were lower in the group with ovarian damage compared to other groups, and atretic follicle numbers were higher. The group with the highest number of primordial, secondary, and antral follicles is the ovarian damage + PRP group. Primary follicles were the highest in the control group. Histopathological changes in the follicles are lower number of ovaries, changes in granulosa cell layers and cytoplasma/nucleus damage. The following features were used in the classification of follicles:

Atretic follicles: degenerated oocyte pycnotic granulosa cells.
Primordial follicles: Single row of squamous granulosa cells surround the oocyte.
Primary follicles: Cuboidal granulosa cells (single layer)
Secondary follicles: Cuboidal granulosa cells (multiple layers).
Antral follicle: There is an antrum in the granulosa cell layers (Pedersen, T. *et al*., 1968).

The images obtained as a result of the histopathological examination are shown in figure 1. Follicles are painted in different forms. In the control and ovarian damage + PRP groups, the follicles were both strongly stained, while weakly stained follicles were observed in the ovarian damage group. In damaged ovarian tissue, increased fibrosis and adhesion, decreased ovarian size remarked.

Table 2: Correlation Between Control, Ovarian Damage, PRP, and Ovarian Damage + PRP*

	Control	Ovarian Damage	PRP	Ovarian Damage and PRP
Control	1			
Ovarian Damage	-.590	1		
PRP	-.225	.271	1	
Ovarian Damage and PRP	.419	-.622*	.134	1

*Correlation is significant at the 0.05 level (2-tailed).

DISCUSSION

In our study, the highest AMH concentration was measured only in the group given PRP, and the lowest value was measured in the group with ovarian damage. The results of Bcl-2 mRNA expression analysis by RT-PCR also showed that the lowest expression occurred in the group with ovarian damage. The correlation analysis results showed a statistically significant, moderate negative correlation between the ovarian damage group and the ovarian damage + PRP group. These results are significant because they show that PRP use improves ovarian damage. Although there was no difference in follicle count results between the

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groups, primordial, primary, secondary, and antral follicle counts were lower, and atretic follicle counts were higher in the group with ovarian damage.

The mechanism of the effects of PRP on regenerative and repair processes is not fully known. In addition, it is known that PRP triggers critical functions in the ovaries by physiological means such as production and release of growth factors, cell growth and differentiation, proliferation, chemotaxis, and angiogenesis. It is thought that PRP induces differentiation of mature oocytes into progenitor cells through signals stimulated. When these mechanisms are examined, it is evaluated that PRP can provide a protective effect on ovarian failure by repairing ovarian damage in Cy-induced POF in the ovaries. PRP can initiate a regenerative process by releasing growth factors, and due to this feature, it may have a protective role when applied together with gonadotoxic agents [Hardwick, J.M. *et al*., 2020-Dehghani, F. *et al*., 2018].

In a study conducted on mice, the ovaries were induced with Cy to produce POF, and the effect of PRP on the restoration of ovarian function was examined. In the study, the effect of PRP was achieved by increasing the release of granulocyte colony-stimulating factors and triggering the mobilization of peripheral mononuclear blood cells. As a result, PRP was induced with Cy and restored ovarian function in rats with POF. According to the data, the ovarian function was improved in the mouse POF model created with Cy. This is important because it shows that PRP has the therapeutic potential to restore ovarian function. The result obtained from the study is similar to that obtained in our study [Pedersen, T. *et al*., 1968]

A study on infertile male rats was conducted by Dehghani et al. Infertility was induced in rats with busulfan, followed by a PRP administration, and the effect of the agent on the structural defect of the rat testis was studied. The results obtained showed that PRP positively affected many parameters, especially the spermatogenic stem cell count, in infertile rat models created with busulfan. However, there was no effect on testicular volume, germinal epithelial height, number of Sertoli and Leydig cells, and length of seminiferous tubules. The researchers concluded that PRP could reverse the structural and functional damage of the testis in line with the results obtained. Although this study seems to be different from our research, it contains similarities in terms of being performed on gonad cells. Both studies showed that PRP treatment promoted the numerical increase of germ cells. While it was observed that PRP did not increase the number of Sertoli and Leydig cells, it was determined that it increased the number of spermatogenic stem cells and sperm. Correspondingly to this, it was determined in our study that it increased the number of follicles. Similar to the increase in testosterone, an increase in AMH was noted in our study [Herraiz, S. *et al*., 2018; Fabi, S. *et al*., 2014].

In an animal study similar to our research, the effect of PRP on ovarian reserves was investigated in Cy-induced ovarian failure. Cortex volume, pre-antral follicle number, and oocyte diameter in antral follicle decreased in Cy-treated rats compared to the control group. Estradiol and progesterone levels decreased. Ovarian cortex volume, pre-antral follicle number, and antral follicle diameter increased in the group whose ovarian damage was treated with PRP compared to the control group. The findings obtained from this study are very similar to our study. The difference between the two studies is that AMH levels were measured in our study and estradiol and progesterone levels were measured in the other study. Today, the general acceptance adopted by experts is that the use of AMH will provide more reliable information in studies on this subject [Fabi, S. *et al*., 2014].

In another study conducted on female rats, it was shown that PRP accelerated antral folliculogenesis by stimulating follicular cell proliferation, and as a result, antral follicle diameter expanded and oocyte diameter decreased in the pre-antral follicle. The researchers emphasized that an increase in oocyte diameter in antral follicles under the influence of growth factors is a common phenomenon, but in this case, a decrease in oocyte diameter in the pre-antral follicle was an unexpected finding. In the study, it was found that PRP had no effect on estradiol and progesterone levels. The study determined that PRP application had no effect on the different structures and functions of the ovaries in normal rats. The beneficial effect of PRP appeared only on damaged ovaries. These results support those obtained in our study. Our study found that PRP accelerates antral folliculogenesis by stimulating follicular cell proliferation (Ozcan, P. *et al*., 2020).

The 2-ΔΔCt method is widely used in a quantitative real-time polymerase chain reaction

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(qPCR) data analysis. This method directly uses the threshold cycles generated by the qPCR system, which is considered a convenient method for calculating relative gene expression levels between different samples. Ct levels are inversely proportional to the amount of target nucleic acid in the sample. The lower the Ct level, the greater the amount of target nucleic acid in the sample. In our study, the qPCR method was used to analyze Bcl-2 gene expression. The values obtained from the calculations made with the 2-ΔΔCt method were used at the end of the analysis. Accordingly, it was determined that Bcl-2 gene expression decreased with PRP use [Huang, Q. *et al*., 2019].

B-cell lymphoma-2 (Bcl-2) family proteins regulate programmed cell death. Some family members (Bcl-2 and Bcl-XL) inhibit apoptosis, while others (Bax and Bak) promote cell death. BCL-2 family proteins transmit signals to induce apoptosis. These proteins undergo conformational changes at the onset of apoptosis and increase the permeability of the outer mitochondrial membrane. In this way, mitochondria are divided into smaller units. Changes during apoptosis, such as the release of cytochrome c in mitochondria, activate caspase proteases, which regulate the degradation of dying cells. There are studies in which the result of decreased Bcl-2 gene expression following PRP application in the literature. PRP was used to treat damage caused by oxidized low-density lipoprotein on human umbilical vein endothelial cells in one of them. The results of the study showed that PRP downregulated pro-apoptotic proteins such as Bcl-2 and caspase-3, as well as IL-6 and IL-1. Another study was designed to evaluate the protective efficacy of PRP against γradiation-induced nephrotoxicity. Expression levels of Bcl-2 and caspase-3 proteins were tested in the study. In conclusion, PRP ameliorated oxidative stress and inhibited apoptosis by downregulating Bcl-2 and caspase-3 [Dehghani, F. *et al*., 2019; Ozcan, P. *et al*., 2020].

In our study, it was concluded that ovarian function would improve if rats with Cy-induced ovarian damage were treated with PRP. It has been determined that PRP administration causes down-regulation of Bcl-2. The improvement in ovarian functions may have resulted from this regulation. In our study, it was also observed that PRP administration caused downregulation of Bcl-2 in rats treated with PRP without ovarian damage. This is important in terms of showing that PRP can also be effective in preventing ovarian damage. Correlation analysis results also indicate a statistically significant, moderate negative correlation between the group with ovarian damage and the group with ovarian damage and PRP applied. This supports our findings.

The strength of our research is its holistic examination of the potential protective effect of PRP against ovarian damage by hormonal, histopathological, and gene expression analysis. Apoptosis is induced by PRP. The Bcl-2 gene family also provides the regulation of apoptosis. Therefore, the expression of the Bcl-2 gene was analyzed in our study. The limitation of our study is that different genes related to apoptosis were not analyzed.

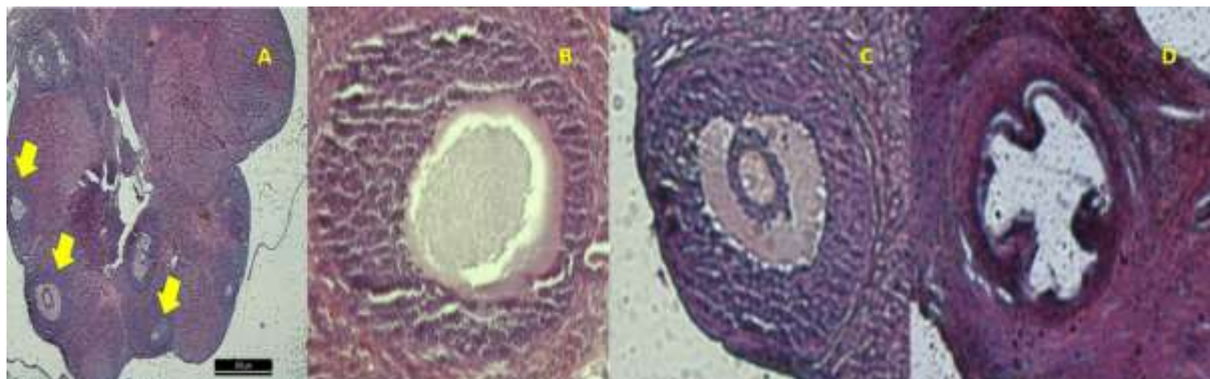

Figure 1: Images obtained as a result of the histopathological examination.
A. Yellow arrows show the different follicles painted in different forms.
B, C, D. Different staining intensity of follicles.

CONCLUSIONS

The basis of PRP's regenerative properties remains unclear. However, the role of growth factors produced from PRP in tissues is critical. These include cell growth, differentiation, proliferation, and processes such as chemotaxis and angiogenesis. Growth factors can repair damage to the ovary. By making use of this feature of PRP,

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follicle repair can be achieved after chemotherapy. It can preserve ovarian function or restore impaired function in Cy-induced POF.

As a result, it is evaluated that PRP can be used as a preventative in women who are treated with Cy and similar agents and subsequently develop ovarian side effects that are very difficult to treat. PRP can also be used to increase the number of follicles. Subjects such as PRP dose, possible side effect profile and duration of treatment can be examined with planned studies. Data from studies on experimental animal models cannot be directly generalized to humans. For this reason, it is necessary to verify the data obtained from animal models on humans with studies to be designed.

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