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Original Research

Reduction of Apoptotic Gene Expression by Platelet-rich Plasma in a Mouse Model of Premature Ovarian Failure

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ABSTRACT

Introduction: Platelet-rich plasma (PRP) has been proposed as a therapeutic intervention for regenerating impaired ovarian tissue and improving outcomes in women with premature ovarian failure (POF). One characteristic of POF is the upregulation of proapoptotic genes such as P53 and Caspase3, which lead to the apoptosis of granulosa cells and monocytes. This study aims to evaluate the effects of PRP on reducing the expression of P53 and Caspase3 genes. **Methods**: A total of 24 adult female Syrian mice aged between 6-8 weeks were utilized in this study. The mice were randomly assigned to four groups: Control, POF, POF+saline, and POF+PRP. Eighteen mice were treated with cyclophosphamide to induce ovarian failure for a duration of 15 days, which was confirmed by vaginal smear. In the POF+PRP and POF+saline groups, 200 μl of PRP and 200 μl of 9% normal saline were injected into the ovaries of the mice, respectively. After two weeks, both ovaries of each mouse were surgically removed for analysis. **Results**: Histopathological analysis revealed a decrease in primary, secondary, and antral follicles in the POF group compared to the control group. Notably, the counts of primary and antral follicles in the POF+PRP group were significantly higher than those in the POF group ($P < 0.05$). Additionally, the number of atretic follicles increased in the POF group but decreased in the POF+PRP groups, although the differences were not statistically significant. The expression levels of Caspase3 and p53 genes were significantly reduced in the POF+PRP group compared to the control group ($P < 0.001$). **Conclusion**: The findings of this study suggest that PRP treatment can effectively restore ovarian function by inhibiting apoptosis in a mouse model of ovarian failure.

Key words: Platelet-rich plasma, Premature Ovarian Failure, Caspase 3, P53, Apoptosis

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INTRODUCTION

Premature (or primary) ovarian failure (POF) is a complex disease characterized by hypoestrogenism, hypergonadotropic, and amenorrhea, ultimately leading to infertility. Individuals with POF are at increased risk of developing various health conditions, including heart disease and osteoporosis. The exact cause of POF remains unknown due to the involvement of multiple factors affecting follicular storage[1]. Current treatment options for POF include hormone therapy, stem cell therapy, and more recently, the utilization of platelet-rich plasma (PRP). The efficacy of PRP is reliant on the abundance of growth factors present in platelet alpha granules, which play crucial roles in regulating follicular migration, extracellular matrix regeneration, cell proliferation, apoptosis, and angiogenesis.

Platelets, blood cells with an average lifespan of around 10 days, serve dual roles in the body: facilitating clot formation during bleeding and releasing growth factors and other proteins[2]. The latter function, not restricted to hemostasis, involves platelets infiltrating body tissues via endocytosis in the extremities and secreting growth factors to support tissue maintenance and regeneration[3]. In response to injury, platelets are among the initial cells to initiate tissue repair processes by releasing various mediators crucial for regeneration. Studies indicate that PRP contributes to the growth and proliferation of various cell types[4].

The beneficial effects of PRP are attributed to the growth factor-containing granules within platelets[5]. Enriched platelets, growth factors, and chemokines stimulate stem cell migration towards tissues, facilitating tissue regeneration[6]. PRP is essential for regulating migration, extracellular matrix regeneration, cell proliferation, and angiogenesis[7]. As over 99% of ovarian follicles degenerate during reproductive life due to postnatal follicular atresia, a small number of follicles persist in menopausal women's ovaries[8].

Apoptosis is a critical process throughout follicular development stages, especially during the transition from preantral follicles to antrum formation[9]. It significantly impacts ovarian follicle fate and fertil-

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ity[10,11]. Caspases, vital enzymes capable of breaking down essential protein substrates within dying cells, play a crucial role in the apoptotic cascade. Caspases require proteolytic processing to become active after being produced as inactive zymogens. Studies reveal the presence of caspase-3, a key executioner caspase, in both the cytosol and mitochondria. Hsp60 and Hsp10, heat shock proteins within the mitochondria, interact with caspase-3 on the inner and outer mitochondrial membranes. p53, a tumor suppressor protein, predominantly localizes to mitochondria following genotoxic stress, where it interacts with various Bcl2 family members to trigger apoptosis[12]. Previous research has indicated the effects of PRP in reducing apoptosis, such as in muscle injury, burns, and osteonecrosis[8,13–15]. Therefore, this study aims to explore the impact of PRP on enhancing ovarian function by reducing the expression of pro-apoptotic genes in a mouse model of premature ovarian failure.

METHODS

Study design

In this study, 24 adult Syrian female mice with an age between 6–8 weeks were used. The animals were kept in a 12-hour dark/light cycle at 25 °C in the animal house of the medical school. All tests were in agreement with the Animal Ethics Guidelines of Shahid Sadoughi University of Medical Sciences (IR.SSU.MEDICINE.REC.1400.017). Animals were divided into four groups (n=6): 1) Control group, 2) POF+Saline group: POF mice which received 9% normal saline solution in their ovaries, 3) POF group: Female mice that received 50 mg/kg of cyclophosphamide solution by daily IP injection for 15 days, and 4) POF+PRP group: POF mice which received PRP in their ovaries (**Figure 1**).

Preparing a POF mouse model

The POF group received 50mg/kg cyclophosphamide as intraperitoneal injection for 15 days. To confirm ovarian failure, vaginal smears were performed on all groups.

Preparation of smear sample from vagina

The reproductive cycle in mice is 4 to 5 days and consists of four stages: proestrus, estrus, metestrus, and diestrus. In the proestrus stage, round nucleated cells are observed. In the estrous stage, most of the cells are without nuclei. In the metestrus stage, there are three types of nucleated epithelial cells, leukocytes, and horn cells. Finally, in the diestrus stage, the predominant cells are leukocytes (**Figure 2**). To investigate the reproductive cycle, 50μL of 0.9% sterile normal saline was gently injected into the vagina, then the samples were placed on a glass slide and observed under a microscope.

PRP preparation

PRP was prepared manually from 10 male mice of the same age. About 2.5-3 ml of blood was taken and immediately transferred to a tube containing an anticoagulant (sodium citrate), with a blood-to-sodium citrate ratio of one-to-nine (1:9). The samples were centrifuged at 2000 g for 10 min. The buffy coat was transferred to a sterile cone microtube and centrifuged at room temperature at 800 g for 10 minutes.

PRP injection

The mice were anesthetized with 10 mg/kg xylazine and 90 mg/kg ketamine. In the POF+PRP group, 200 μl of fresh PRP was injected into each ovary, and the rest was used for injection into the adjacent tissues and the abdomen.

Tissue separation

Two weeks after the PRP injection, ovaries were removed and one of them was randomly placed in 10% formaldehyde for histological studies. Another ovary was used for molecular studies.

Histological Findings

Formalin 10% was used for the fixation of ovarian tissue. After tissue processing and preparation of blocks, thin sections of 5-6 microns were prepared. Ovarian tissue was stained with H&E. Finally, tissue samples were observed and graded by a light microscope[13].

Gene Expression Analysis

RNA extraction was performed using the RNX-Plus RNA extraction kit (SinaClon, Iran). In summary, after separating 30-50 mg of tissue sample, 1 ml of RNX-Plus was added to each sample and homogenized. After adding 200μl of chloroform and shaking for 15 seconds, the sample was placed on ice for 5 min. Centrifugation was performed at 12000 g and 4 °C for 15 min. One third of the upper aqueous phase containing the RNA was transferred to a new microtube. Then, the same volume of cold isopropanol was added and placed on ice for 15 min. Centrifugation was carried out at 12000 g and 4 °C for 15 min. The supernatant was drained, and 1 ml of 75% cold ethanol was added. The centrifugation was performed at 7500 g

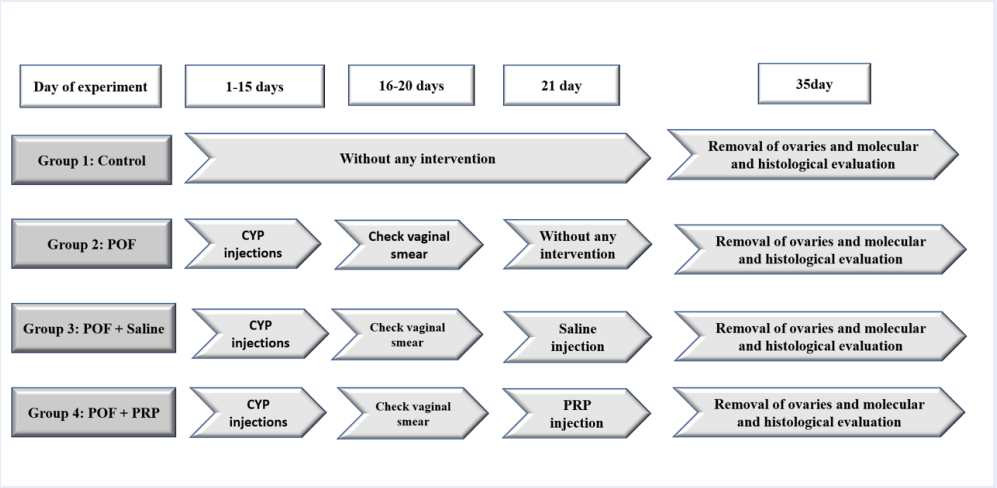

Figure 1: **Schematic representation of timeline and groups used for evaluate effect of plasma rich plasma (PRP) in premature ovarian failure (POF) in mice model induced by the cyclophosphamide (CYC).**

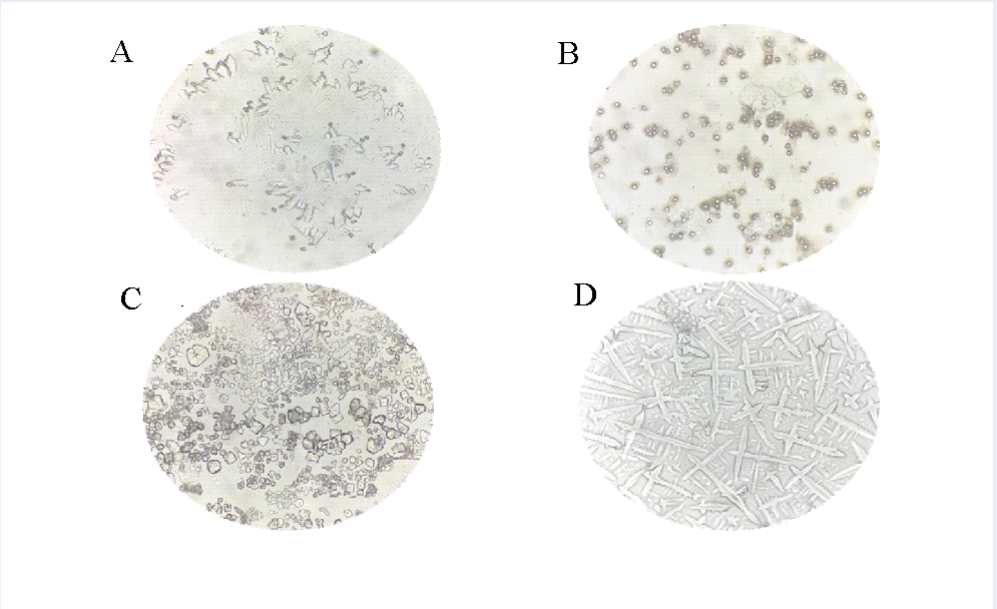

Figure 2: **Reproductive cyclin mice A) Proestrus: round nucleated cells are observed, B) In the estrous stage, most of the cells are without nuclei, C) In the metestrus stage, there are three types of nucleated epithelial cells, leukocytes, and horn cells and D) in the diestrus stage, the predominant cells are leukocytes.**

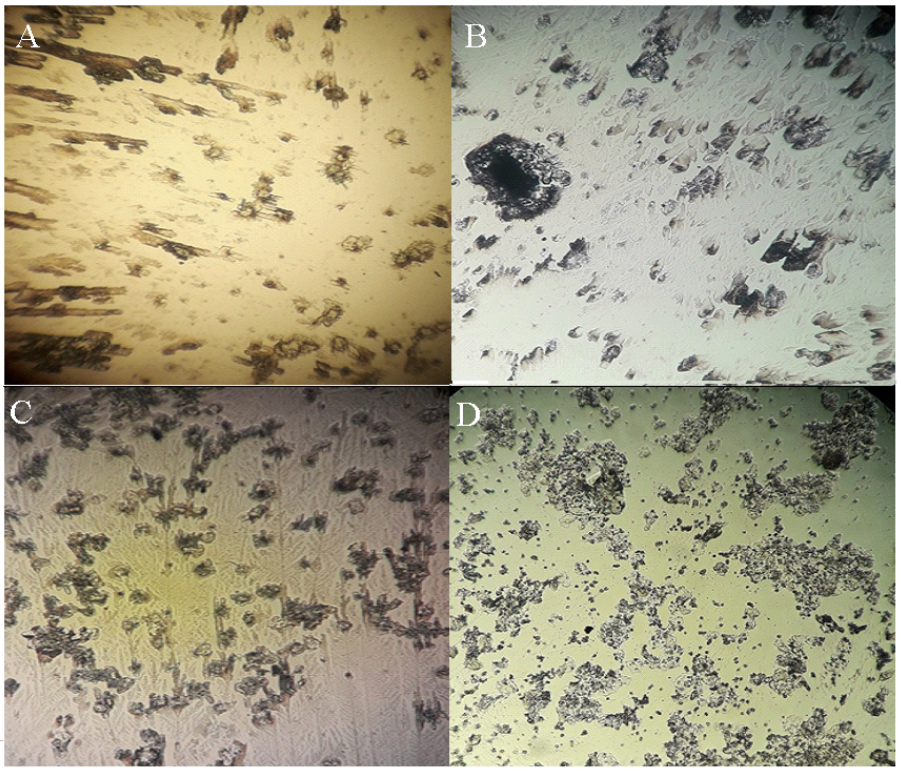

Figure 3: **Representative of smear samples prepared from mice vaginal fluid during 4 days for To confirm premature ovarian failure (POF) model**. Images **A**) 1 day, **B**) 2 days, **C**) 3 days, and **D**) 4 days show a stop in the estrous cycle.

Table 1: Sequence of primers used to perform real-time RT-PCR reaction

Primer name	Sequences (5'-3')
p53F	TCTTCTGTACGGCGGTCTCTC
p53R	ACCGCCGACCTATCCTTACC
GapdhF	TCCACGACATACTCAGCAC
GaphdhR	TCCACGACATACTCAGCAC
CAsp3F	GGGACGCTTGGAACGGTACG
CAsp3R	CCACTGACTTGCTCCCATGT

F: Forward, **R**: reverse

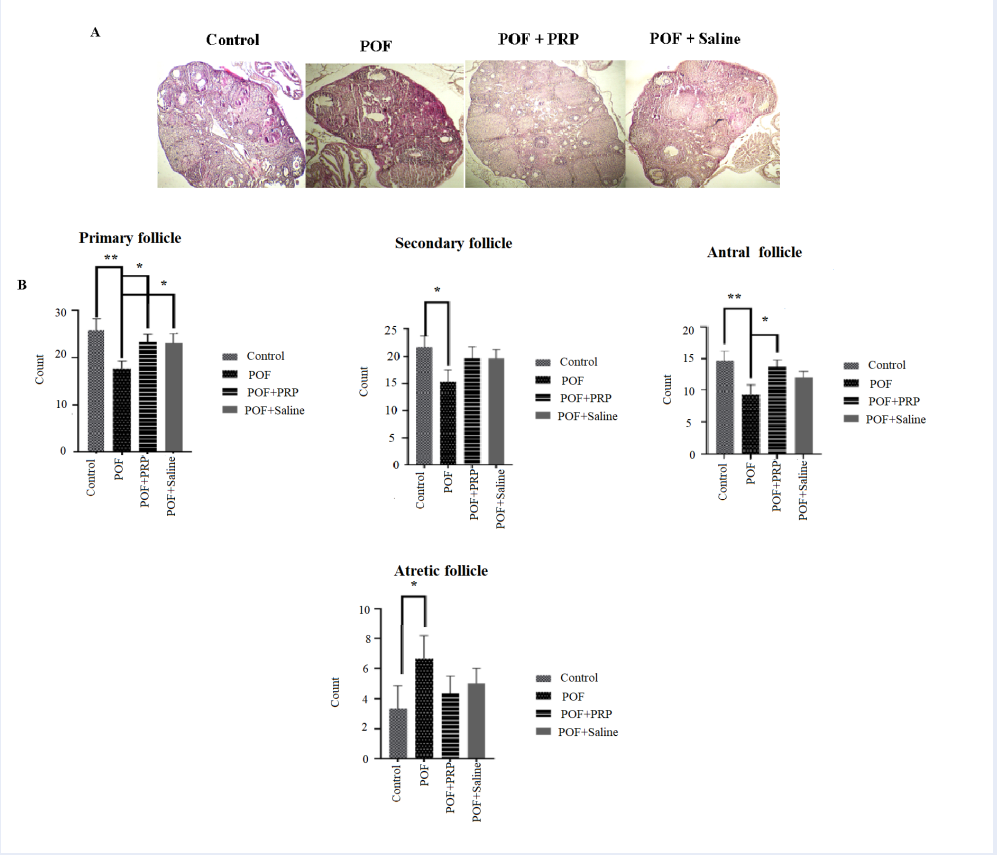

Figure 4: **Effect of plasma rich platelet (PRP) on ovarian morphology and follicular development in the POF model**. **A**) Representative macroscopic images of effect of PRP on ovarian tissue after hematoxylin and eosin (H&E) staining of ovarian in control group and premature ovarian failure (POF) group and POF mice which received PRP in and in POF mice which received 9%normal saline solution (100 x magnification. Scale bars: 200 μm). **B**) Quantitative measurement of the number of primary, secondary, antral, and atretic follicles in the control and experimental groups (**P < 0.01 and *P < 0.05)

and at 4 °C for 5 min. Finally, 50 DE1 DEPC treated water was added to the RNA precipitate.

cDNA synthesis was performed by the Pars Toos company kit according to the manufacturer's protocol. The primers for the P53, Caspase, and GAPDH genes (**Table 1**) were prepared and cDNA was amplified by Real-time PCR using SYBR Green dye.

Statistical Analysis

The gene expression analysis was performed using GraphPad Prism software. The significance of the difference between groups was analyzed by One-way ANOVA and Tukey tests (P < 0.05).

RESULTS

Results of vaginal fluid smear examination showed that the estrous cycles of mice in the control group were regular (rhythmic cycles occurred every 4-5 days and consisted of four regular and continuous phases), while those in the POF groups were disordered and irregular throughout the experimental period. Abnormal rates of estrous cycles and estrous frequency were observed in all POF groups, with either prolongation, stagnation, or no obvious estrus cycles (**Figure 3**).

Evaluation of ovarian tissue

Histopathological study revealed that the POF group had significantly fewer primary, secondary, and antral follicles compared to the control group. Additionally, the POF group that received PRP showed significantly more primary and antral follicles than the POF group without PRP (P < 0.05) (**Figure 4A&B**). While the number of atretic follicles was higher in the POF group compared to POF+PRP, these changes were not significant.

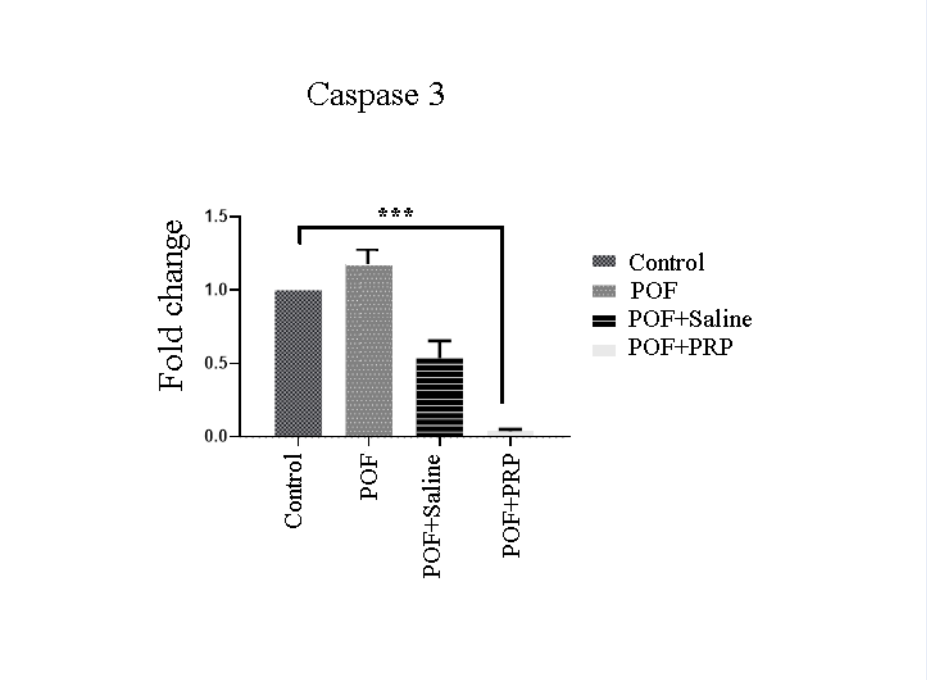

Figure 5: **Effect of plasma rich platelet (PRP) on the expression of the gene associated with apoptosis (*Caspas3* gene) in ovarian tissues**. The mRNA expression level of *Caspas3* gene was measured using qRT-PCR in control group and premature ovarian failure (POF) group and POF mice which received PRP in and in POF mice which received 9% normal saline solution and normalized to GAPDH. Data are represented as means ± SEM from three independent experiments. (***P < 0. 001)

Molecular evaluation of ovarian tissue

Compared to the control group, POF mice receiving PRP had significantly lower Caspase3 gene expression ($P < 0.001$). Moreover, there was a significant increase in Caspase3 gene expression in the POF group compared to the control group ($P < 0.05$). In the intergroup study, the Caspase3 gene was significantly downregulated in the POF group that received PRP compared to the POF group without PRP ($P < 0.001$) (**Figure 5**). The expression level of the P53 gene was significantly lower in the POF mice that received PRP compared to the control group ($P < 0.001$), and significantly higher in the POF group without PRP compared to the control group ($P < 0.01$). Additionally, POF mice treated with PRP showed considerably lower P53 expression compared to the control group ($P < 0.001$) (**Figure 6**).

DISCUSSION

The ovary naturally loses several follicles after birth. The remaining primordial follicles are inactive until puberty. After puberty, most germ cells in the atretic pathway are destroyed; thus, few cells remain in the ovary [13]. In rodents, such as mice, more than 99% of ovarian germ cells are killed by atresia [14].

Ovarian failure is defined as ovarian dysfunction before 40 years old. In this state, the remaining follicles in the ovary are inactivated at a young age and during the process of apoptosis or inhibition of reactivation of ovarian germ cells [15]. Acute ovarian failure can occur after chemotherapy or radiotherapy, which can be transient or permanent. The term failure means abnormal ovarian function, although it does not imply that the ovary function is permanently severed. Patients diagnosed with ovarian failure may have intermittent estrogen secretion and ovulation, and in 5-10% of cases, pregnancy spontaneously may occur. Therefore, according to the cause of ovarian failure, ovarian function can be permanent or temporary [16]. Since most chemotherapy drugs affect dividing cells, it is believed that they will suppress the growing cells in the ovary. The ovaries of women have a normal or slightly reduced number of primary follicles during chemotherapy, while the number of larger mature follicles is further reduced, indicating a greater effect of chemotherapy drugs on the development and maturation of these follicles [17]. Alkylating drugs, such as cyclophosphamides, are the most well-documented and

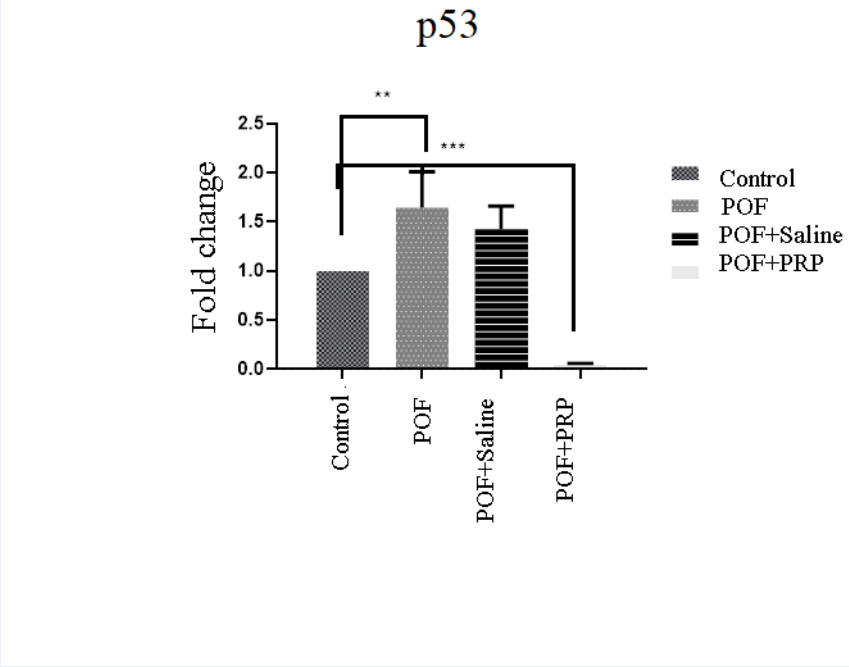

Figure 6: **Effect of plasma rich platelet (PRP) on the expression of the gene associated with apoptosis (*P53* gene) in ovarian tissues.** The mRNA expression level of *p53* gene was measured using qRT-PCR in control group and premature ovarian failure (POF) group and POF mice which received PRP in and in POF mice which received 9% normal saline solution and normalized to GAPDH. Data are represented as means ± SEM from three independent experiments. (***P < 0. 001 and (**P < 0.01).

potentially harmful drugs which lead to ovarian failure[18]. In this study, it was detected that the number of primary, secondary, and antral follicles in the POF mice treated with cyclophosphamide was significantly lower than the control group.

When exposed to cyclophosphamide, the apoptosis process plays an important role in reducing the number of follicles[19]. In fact, cyclophosphamide can induce apoptosis by producing ROS and oxidative stress in ovaries[20]. A study showed that in POF mice, the expression of anti-apoptotic and pro-apoptotic proteins such as Bcl-2 and Bax were significantly reduced and increased, respectively[21]. Also, in many cancer models induced by cyclophosphamide, it was shown that the high production of ROS increases caspase 3 in ovarian follicles, and ultimately leads to follicular diminution. Furthermore, DNA fragmentation occurs in ovarian tissue cells after using cyclophosphamide[19]. In the current study, the expression levels of pro-apoptotic genes of p53 and Caspas3 were significantly increased in the POF group which indicated high apoptosis in the ovary.

PRP was first discovered by Marks *et al.*[22]. Numerous studies have shown that the beneficial effects of PRP are due to the presence of various growth factors in alpha-platelet granules[23]. Also, by stimulating chemotaxis, PRP improves differentiation, proliferation, and angiogenesis of stem cells, finally leading to tissue repair[24].

According to the role of mesenchymal stem cells and PRP in the repair of damaged tissue, they have been used in the treatment of many diseases. Local regeneration and angiogenesis are the effects of PRP, which have been confirmed in numerous studies on several tissues including bone, tendon, muscle, skin, and cartilage[25,26]. In addition, since the preparation of PRP is easy and can be performed at a low cost, it is widely employed in research[27,28]. PRP injection into the ovaries can improve ovarian function and act as a regenerator. This product has good effects on ovarian failure by releasing known growth factors[29].

In the present study, it was observed that in the group with ovarian failure which received PRP as treatment, the number of primary and antral follicles was significantly increased compared to the POF group, and

also the count of secondary and antral follicles was increased compared to the POF group. However, these changes were not statistically significant.

Also, the expression level of pro-apoptotic genes of *p53* and *Caspas3* in the POF plus PRP group had a significant decrease compared to the POF mice which received no treatment, indicating the effect of PRP on improving ovarian function.

Application of PRP for the treatment of androgenic alopecia showed that PRP considerably increased gene expression including *β-Catenin, AKT,* and *PDGF*, and suppressed *p53* gene [30].

Another study focused on autologous PRP and MSCs isolated from rat uterus and compared them with the effects of autologous ordinary plasma (OP). In this study, after PRP exposure, expression of the *p53* gene remained unchanged, and relative Bcl-2 production did not differ significantly among the studied groups [31].

This study demonstrated that the number of primary, secondary, and antral follicles was increased in the POF plus saline group. This can be due to the effect of mechanical puncture on ovary function. Studies have shown that damage to the ovary can cause the activation of the Hippo and the Akt pathways which play a role in the activation of primary follicles [32,33]. In this study, considering the improvement of ovarian function after saline injection, it is suggested to conduct a comparative study between the effect of platelets and the effect of mechanical mechanisms that stimulate the function of the ovary.

Although this study identifies a reduction in the expression of apoptotic genes in the mice model of POF induced by cyclophosphamide, it does not fully address the underlying mechanisms by which PRP affects these changes at the molecular or cellular level. Therefore, further studies can clarify the pathways involved in the observed effects. On the other hand, as regarding a typical sample size for preliminary animal studies is 5-10 mice in each group, larger sample sizes might be needed to decrease the variability and increase the confidence in the findings, particularly for making robust statistical comparisons across multiple groups. Also, the findings of experimental animal models may not directly predict accurate outcomes in human reproduction. So, more research is required to confirm the effects of PRP on the human ovary.

CONCLUSION

Based on the findings of this study, it can be concluded that PRP can be beneficial in reducing apoptosis induced by cyclophosphamide and thereby improving ovarian function in POF. Identifying the pathway that disrupts ovarian function can be useful in discovering a solution for treatment. Also, considering the role of PRP in reducing the expression of apoptotic genes, PRP can also help in the treatment of diseases in which apoptosis is involved.

ABBREVIATIONS

Akt - Protein kinase B, **Bax** - Bcl-2-associated X protein, **Bcl-2** - B-cell lymphoma 2, **H&E** - Hematoxylin and eosin, **IP** - Intraperitoneal, **MSCs** - Mesenchymal stem cells, **OP** - Ordinary plasma, **POF** - Premature ovarian failure, **PRP** - Platelet-rich plasma (2 occurrences), **ROS** - Reactive oxygen species

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AUTHOR'S CONTRIBUTIONS

Elham Dehghan Manshadi performd *in vivo* experiments on mice. Marzieh Lotfi and Mohammad Ebrahim Rezvani designd experiments, checked results, and conducted the research. Azam Hassanpour and Fatemeh Zare Mehrgerdi revised the manuscript. All authors read and approved the final manuscript.

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AVAILABILITY OF DATA AND MATERIALS

Data are available from the corresponding author on reasonable request.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study on animals was approved by Shahid Sadoughi University of Medical Sciences (IR.SSU.MEDICINE.REC.1400.017).

CONSENT FOR PUBLICATION

Not applicable.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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