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INFERTILITY: ORIGINAL ARTICLE

Ovarian Rejuvenation Through Platelet-Rich Autologous Plasma (PRP)—a Chance to Have a Baby Without Donor Eggs, Improving the Life Quality of Women Suffering from Early Menopause Without Synthetic Hormonal Treatment

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Abstract

Due to the fact that modern American and European women postpone childbirth until later in life, they are more likely to face the problem of ovarian insufficiency by the time they are ready to have children. So, the ability to restore the ovarian function safely is crucially important. Our study involved 38 women 31–45 years of age with low ovarian reserves and at least two unsuccessful attempts to receive their oocytes through IVF. The blood from the patients was collected into two BD vacutainers for PRP preparation. The platelet concentration in the PRP was 1×10^6 μl. PRP injections into the ovaries were performed by a gynecologist with a special 25G needle, 20 cm in length, as an ultrasound-guided procedure or a laparoscopic-assisted approach. After PRP treatment, women were tested with several criteria for 12 months. We saw a significant improvement in hormone levels; six healthy babies were born, ten pregnancies were achieved, and four out of the ten were from natural conception. The PRP injections into the ovaries are safe, productive, and a natural treatment that may help women with premature ovarian insufficiency to give birth to their own child. The difference and novelty with our method of ovarian rejuvenation is in obtaining a higher platelet concentration (about 1×10^6 μl), which allows us to achieve long-lasting results, within 12 months, after a single procedure. Additionally, for the first time, we proposed and successfully performed a laparoscopically assisted technique for administering PRP into the ovary.

Keywords Low ovarian reserve · Platelet-rich autologous plasma · Follicle-stimulating hormone · Luteinizing hormone · Anti-Müllerian hormone

Introduction

Modern American and European women postpone childbirth until later in life. Due to this fact, they are more likely to face the problem of ovarian insufficiency by the time they are ready to have children. Moreover, the tendency of the unwanted influence of environmental and social factors increases the incidents of early menopause. To solve the problem, a reproductive assistant can offer the donor egg. Such a solution can pose significant psychological and financial concerns. So, the possibility to restore the ovarian function is crucially important.

Ovarian folliculogenesis is a complex and sophisticated process that can be divided into two main phases—gonadotropin independent and gonadotropin dependent [1, 2]. In early stages, from primordial follicles to preantral follicles, they can develop under the influence of intraovarian factors independently of gonadotropins. Starting from the preantral phase, follicle-stimulating hormone (FSH) influences follicles, and they become dependent upon FSH. The antral stage of folliculogenesis is believed to be a crucial point where the further fate of follicular development is determined. Only a few follicles are selected to grow into a mature form. About 99% will undergo follicular atresia.

Follicular atresia can develop during every stage and reflects the balance between apoptotic and survival stimuli. The deficiency in extracellular survival stimuli can lead to

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apoptosis. In many species, the tumor necrosis factor (TNF) family with its receptor is believed to be the main trigger of apoptosis. The survival of follicles, processes of recruitment, selection, and maturation of follicles are managed by complex intraovarian and systemic signals.

Most of the growth factors that are produced by somatic ovarian cells and oocytes belong to a transforming growth factors (TGF) superfamily. There is a group of bone morphogenic proteins among which BMP-4 and BMP-7 are responsible for the transition from primordial to primary stage. They are secreted by ovarian stromal cells and theca cells. BMP-15 (also known as GDF-9B) and growth differentiation factor-9 (GDF-9) are emitted from the early stage and have a pivotal role in follicle maturation after the primary step. There is also a plethora of growth factors belonging to the TGF superfamily such as glial cell–derived neurotrophic factor (GDNF), Müllerian inhibitory factor (MIF), activin/inhibin, and TGFβ [3].As follicles transform to the antral stage, the influence of gonadotropins becomes crucial. FSH provides further survival and growth.In granulosa cells, the growth factors of the TGF superfamily can influence FSH receptors and modulate functions of FSH. It is shown that some members of the TGF superfamily can stimulate the expression of the follicle-stimulating hormone receptor (FSHR). [4] TGF-β can enhance FSH-stimulated progesterone production and luteinizing hormone (LH) receptor expression [5]. LH and progesterone are capable of suppressing follicular apoptosis [6].

Except for the critical role of the TGF superfamily in the paracrine regulation of folliculogenesis, there are also extensively described insulin-like growth factors (IGFs) and IGF-binding proteins (IGFBPs) that play an essential role in the management of follicular growth and maturation [7].All these factors may be involved in the development of premature ovarian failure (POF). POF is a complex disease, and the mechanisms leading to this condition are mostly unknown. Inactivation of TGF-related factors may contribute a significant part to the pathogenesis of POF [8].

In recent years, considerable attention has been paid to platelet-derived factors. There are numerous studies describing that the alpha granules of platelets contain more than 30 mitogenic and chemotactic growth factors (GFs). These GFs play an essential role in paracrine-mediated wound healing. The alpha granules of platelets contain platelet-derived growth factor (PDGF); transforming growth factors β1, β2, and β3 (TGF-β1, TGF-β2, TGF-β3); platelet-derived angiogenesis factor (PDAF); insulin-like growth factor 1 (IGF-1); platelet factor 4 (PF-4); epidermal growth factor (EGF); epithelial cell grow factor (ECGF); vascular endothelial cell growth factor (VEGF); primary fibroblast growth factor (bFGF); bone morphogenetic proteins BMP-2, BMP-4, BMP-6, and BMP-7; and other cytokines [9, 10].There are several platelet-derived cytokines that are involved in wound healing. The ability of platelets to degranulate and release cytokines is used for platelet-rich plasma (PRP) therapy in many fields of medicine. One new approach for using them in the treatment of premature ovarian insufficiency was suggested not long ago [11]. There are several possible explanations for the reversal of aging ovaries that have been proposed. One of them is the hypothesis about the activation of renewable ovarian germline stem cells (GSCs) by the platelet-derived growth factors [12]. Another possibility is that some amount of dormant primordial follicles is kept in the ovary and the cytokines are present in the platelets—TGF superfamily, IGF-1, BMP-4, and BMP-7 can promote folliculogenesis.Even though successful results after intraovarian PRP injections have been reported, there is still no unified protocol for PRP preparation. We believe that proper platelet concentration and a volume of PRP injected are the key for achieving good and consistent results. In our clinic, we used a two-step protocol for obtaining a platelet concentration of about 1×10^6μl. This amount corresponds to approximately five times than that concentration in the whole blood.

Method

Our study involved 38 women who were 31–45 years old. These were women who had infertility due to a low ovarian reserve and at least two unsuccessful attempts to get their oocytes via IVF cycles in their anamnesis. The selection of patients, procedure, and further monitoring were carried out in the nongovernment medical center "Ultrasound Pro," Kyiv, Ukraine. Before being included in the study, the patients got acquainted and signed an informed consent for the procedure and the publication of the data obtained from the study. Women were tested for pregnancy with negative results, and they did not have a history of any significant chronic condition, cancer, or mental illness. A transvaginal ultrasound scan showed at least one normal ovary with a volume not less than 1 ml and no ovarian or uterine lesions. All patients were examined before the procedure of ovarian rejuvenation: general blood analysis, coagulogram, general urine analysis, cytology of the cervix and cervical canal (PAP smear), blood tests for hepatitis B and C, RW, HIV1/2, electrocardiogram, FSH, LH, estradiol, and AMH. Also, women filled out a form with information about their self-life satisfaction, the regularity of the menstrual cycles, and desire to have a baby before the procedure. Moreover, they were asked to fill up the same form again 1, 2, 3, 6, and 12 months after ovarian rejuvenation.

PRP Preparation

Blood was collected from the patients into two BD vacutainer ACD-A tubes in the proportion of 8.5 ml of the blood and 1.5 ml of trisodium citrate with citric acid and dextrose. After centrifugation at a G-force of 800 for 3 min, the platelet-poor plasma (PPP) was achieved. The PPP was then withdrawn and

transferred into Falcon 15-ml conical centrifuge tubes. After centrifugation for 15 min at room temperature at a G-force of 1400, the precipitate of platelets was obtained, and then 75% of the upper volume of PPP was withdrawn again. The platelet precipitate was resuspended in the remaining PPP. Therefore, platelet-rich plasma was produced in the amount of 2 ml; 0.7 ml of PRP was injected into each ovary with a concentration of 1,000,000 platelets per microliter (μl). This corresponds to 700,000,000 platelets per ovary.PRP injections into the ovaries were performed by a gynecologist with a special 25G needle, 20 cm in length, as an ultrasound-guided procedure. In difficult cases, when it was impossible to reach the ovaries vaginally, or the goal was to check the tubal patency test, a laparoscopic-assisted approach was used (Figs. 1, 2).

Safety Parameters

The procedure for ovarian rejuvenation can have some complications, such as penetration into the blood vessels or intestines. However, the ovarian rejuvenation procedure is generally safe provided that an experienced gynecologist has excellent ultrasound navigation skills. Transvaginal access is the same as for egg collection during IVF. The use of color Doppler helps to avoid damage to potentially dangerous large blood vessels. If there are some unavoidable obstacles, such as abnormal localization of the ovaries, large fibroids, intestines, or blood vessels, laparoscopic access is used to ensure safety. A careful screening and selection of patients is performed to avoid possible complications. Only those who have at least one visible ovary can be included in the procedure. Other routine tests that are used before the method are the same as the usual ones before any operation of this type.

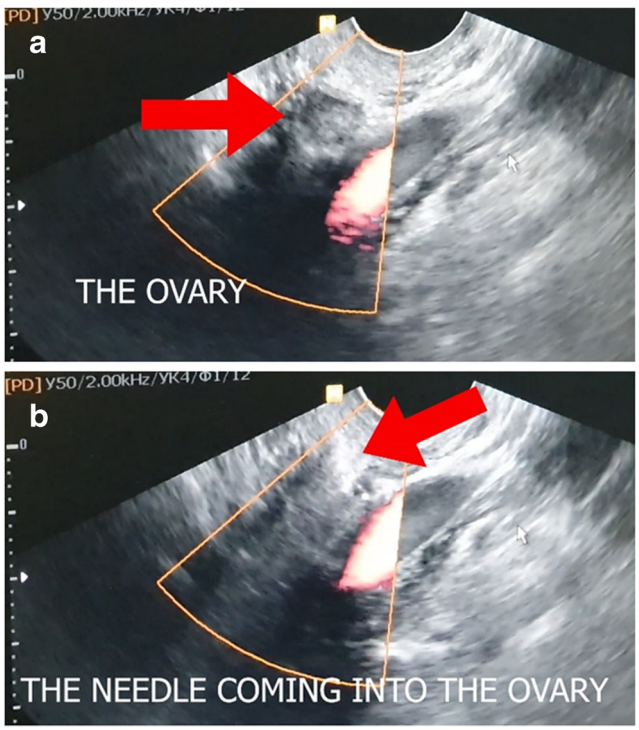

Fig. 1 Ultrasound-guided ovarian rejuvenation procedure. **a** The needle is going through the vaginal wall, puncturing the ovarian cortex, and **b** enriched plasma is coming into the ovary

Assessment

After PRP treatment, women were tested with several criteria, which were divided into objective and subjective outcomes, for up to 12 months. We consider the serum levels of hormones, delivering a baby, pregnancy rates, and own oocytes retrieved via IVF as objective results. Also, the women's self-satisfaction, their assessment of menstrual cycle regularity, and desire to have children denote subjective outcomes.

Objective outcomes

1. Serum LH, FSH, AMH, and estradiol levels were analyzed after 1, 2, 3, 6, and 12 months. This data was statistically processed.
2. Delivery of a baby, pregnancy rate, and own oocytes retrieved.

Subjective outcomes

1. A regular menstruation
2. Self-satisfaction. Improving vasomotor episodes, sexual life impact, emotional stability, achieving desired weight, and so on
3. Wish to have a baby

Statistical Analysis

All calculations were performed with Statistica (data analysis software system), version 13. We used a one-way ANOVA test for comparison and Tukey post hoc test for FSH, LH, estradiol, and AMH levels directly before the PRP procedure as well as in 1 month, 2 months, 3 months, 6 months, and 12 months post-procedure, where $p < 0.05$ was accepted as statistically significant.

Results and Discussion

The levels of the hormones FSH, LH, estradiol, and AMH were analyzed using the one-way ANOVA followed by Tukey-Kramer multiple comparisons test.

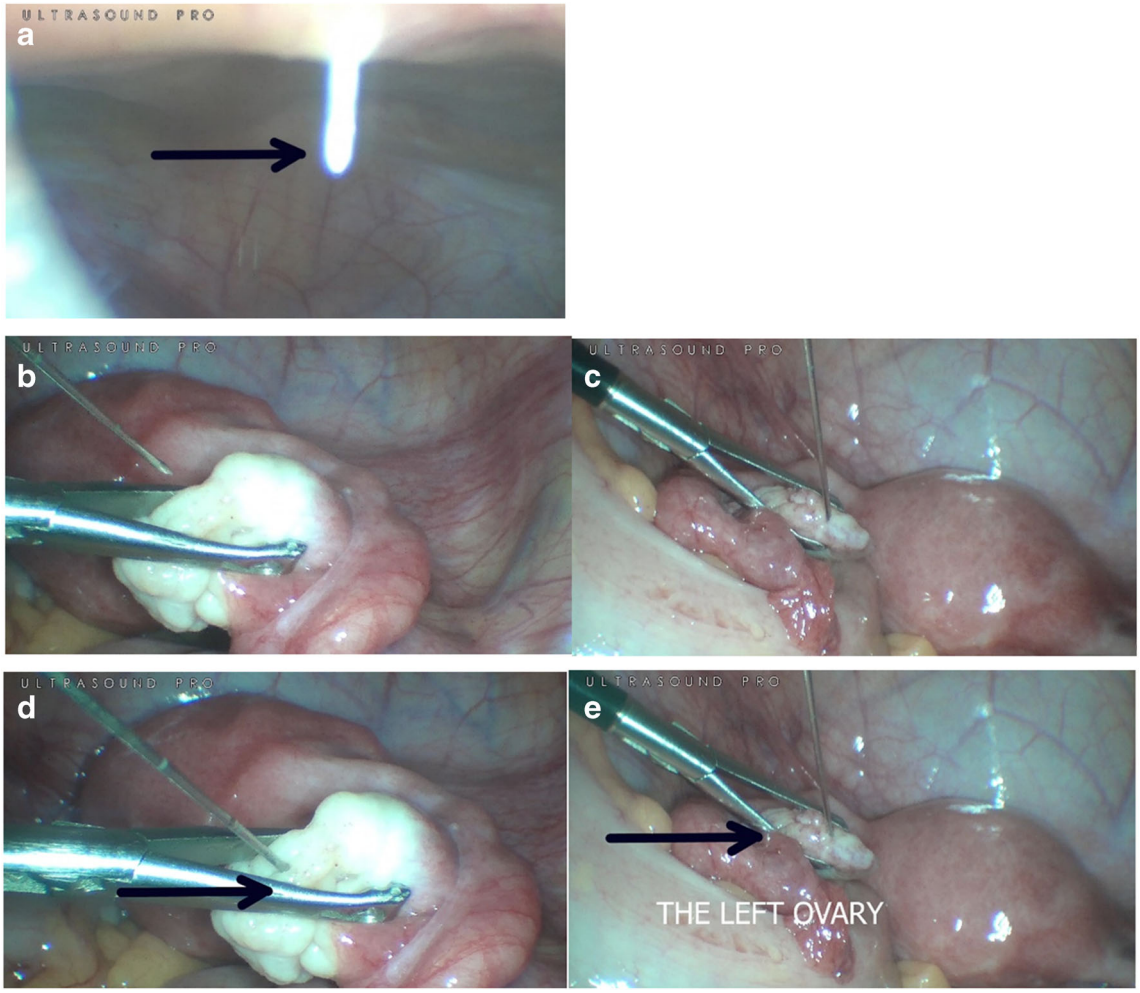

Fig. 2 **a**–**e** PRP into the ovary with the laparoscopic approach. **a** The needle is going through the abdominal wall; **b**, **c** puncturing the ovarian cortex; and **d**, **e** enriched plasma is entering the ovary

It is noteworthy that the level of FSH and LH decreased in a month and remained significantly lower throughout almost the entire study, for the 2nd, 3rd, 6th, and 12th months following the introduction of PRP, compared with the group before the treatment ($p < 0.0007$–0.00004) (Figs. 3, 4). It is likely that PRP thus exhibits its regulatory and immunomodulatory abilities.

However, the level of estradiol, after PRP treatment, steadily increases from the 1st till 6th month. Then, it dropped slightly at 12 months. Compared with results from before

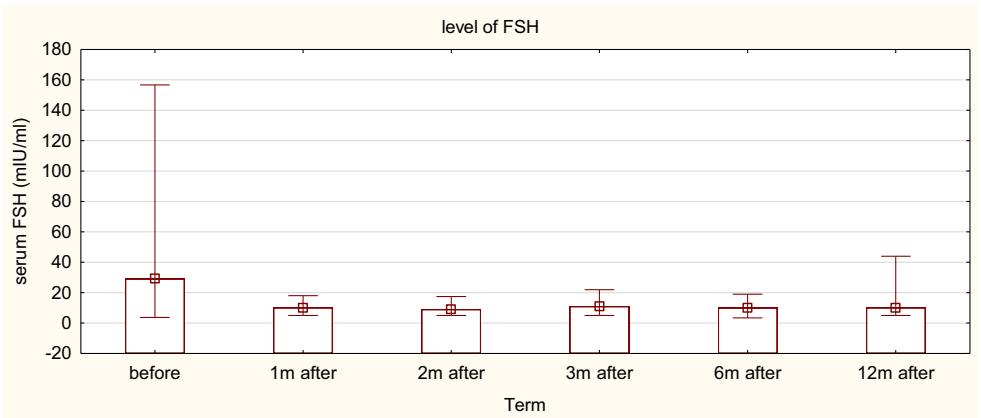

Fig. 3 The level of FSH during the study before the ovarian rejuvenation and after the procedure. One-way ANOVA followed by Tukey-Kramer multiple comparisons test, $p < 0.05$

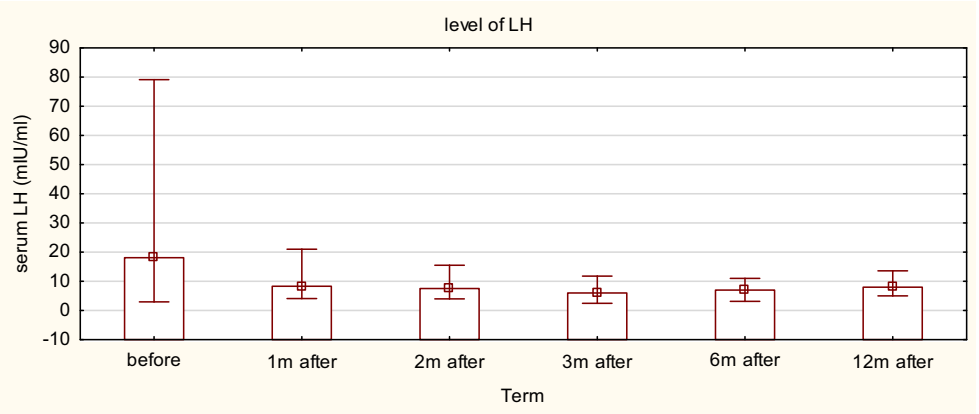

Fig. 4 The level of LH during the study before the ovarian rejuvenation and after the procedure. One-way ANOVA followed by Tukey-Kramer multiple comparisons test, $p < 0.05$

rejuvenation, the most significant levels of estradiol were achieved at the 6th and 12th months ($p < 0.0003$; $p < 0.00005$) (Fig. 5). Such data may indicate that growth factors from platelets improve the microenvironment for sleeping follicles, and they could grow in response to the PRP treatment [].

From the first view, the dynamics of AMH did not allow us to draw a conclusion about the benefits of PRP, as the chart below illustrates. Nevertheless, according to statistical calculation, we got justification for the increase in AMH level at 12 months after rejuvenation, in contrast with the results from before the procedure ($p < 0.000023$) (Fig. 6).

Many women have shown dramatic improvement of AMH level up to 1.1 ng/ml compared with 0.08 ng/ml before the ovarian rejuvenation. In contrast, there were some women with low AMH during the entire study, but it did not prevent them from getting pregnant or at least allowing the retrieval of their eggs.

During our study, after ovarian rejuvenation, four women became pregnant on their own (natural conception) and gave birth to healthy children. Twenty women underwent IVF with a successful sampling of one—three eggs in fifteen of them. Consequently, five women had empty follicle syndrome, the probable occurrence of which is from 0.045 to 7% in IVF cycles according to various sources [13, 14] (Fig. 7). For eight women, the embryos were transferred in the same menstrual cycle. In seven cases, the embryos were frozen due to the insufficient quality of the endometrium for implantation or other reasons.

Out of eight transfers, positive beta-hCG and visualization of the embryo with a heartbeat were obtained in five cases; four women successfully passed the first screening. Two women gave birth to healthy children, and two pregnancies at the time of writing were uneventful (second and third trimester). Consequently, one pregnancy miscarried at 6 weeks. A genetic study of abortus confirmed a normal karyotype.

In a group of seven frozen nuclei, two revealed genetic abnormalities, and the other five were implanted. Positive beta-hCG and visualization of the embryo with a heartbeat were obtained in 2 cases; two women had completed the first screening with average results at the time of this writing (Fig. 8).

The data obtained during a survey of women after the ovarian rejuvenation procedure, reflecting subjective outcomes, are presented in Fig. 9. It should be noted that the desire to have children had decreased by the end of the study, so that, in our opinion, it affected the number of pregnancies because some women took steps to prevent pregnancy (Fig. 9).

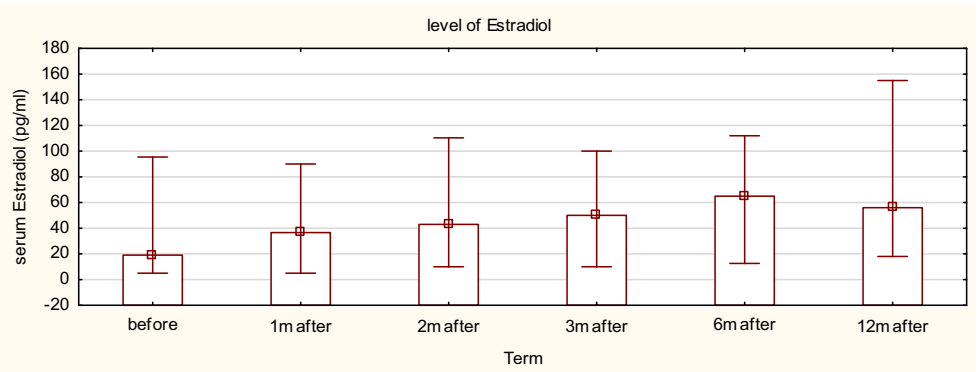

Fig. 5 The level of estradiol during the study before the ovarian rejuvenation and after the procedure. One-way ANOVA followed by Tukey-Kramer multiple comparisons test, $p < 0.05$

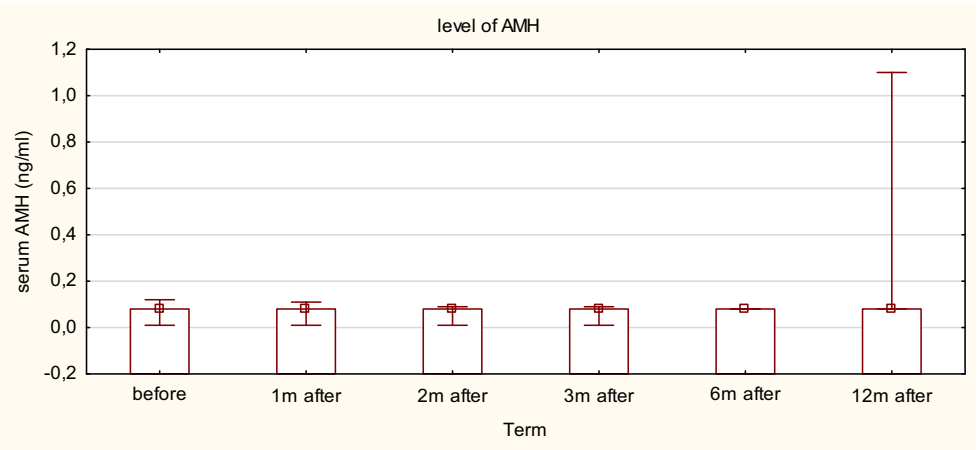

Fig. 6 The level of AMH during the study before the ovarian rejuvenation and after the procedure. One-way ANOVA followed by Tukey-Kramer multiple comparisons test, $p < 0.05$

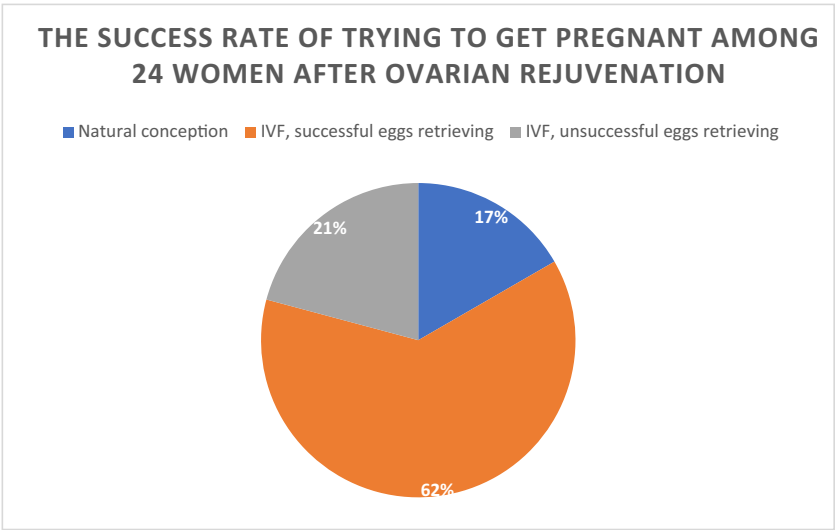

Fig. 7 The pie chart shows the proportion of natural conception (blue), egg retrieval (orange), and empty follicles (gray) after the ovarian rejuvenation

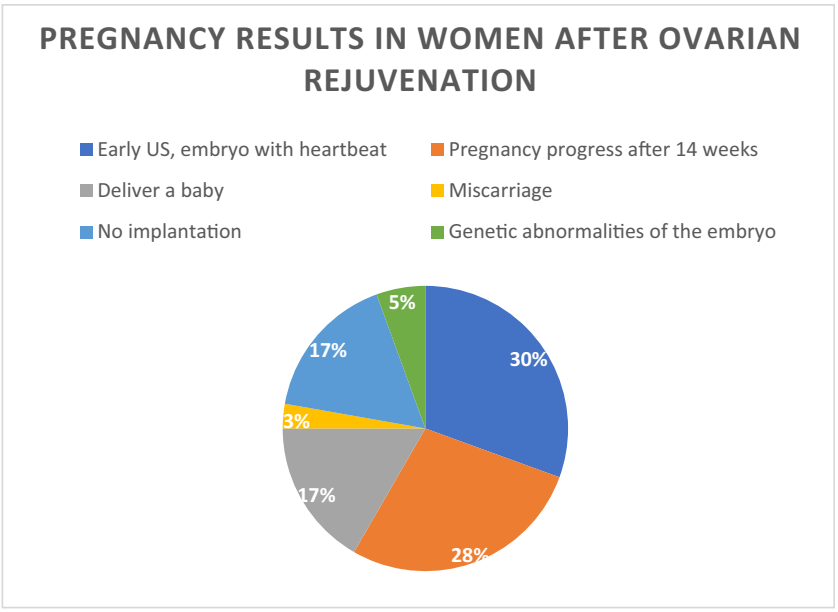

Fig. 8 The pie chart shows the proportion of pregnancy outcomes after PRP injection into the ovaries

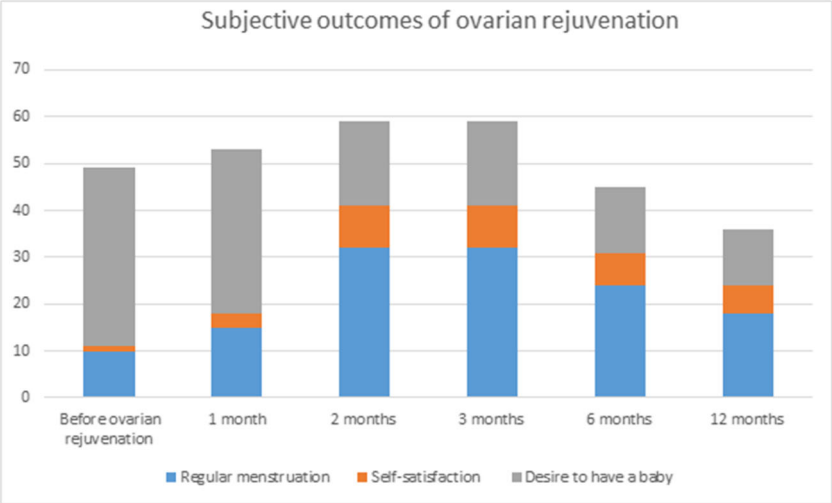

Fig. 9 The bar chart indicates the proportion of self-satisfaction, desire to have a baby, and regular periods among women after PRP injection into the ovaries

Conclusions

Injection of PRP into the ovaries gave women a chance to have a baby with their own eggs. It allowed them to mitigate the severity of the symptoms of early menopause. The facts were proven with the improvement of FSH, LH, estradiol, and AMH hormone levels, achieving the desired pregnancy as well as giving birth to healthy children. So, the PRP can be used as an isolated therapeutic tool for women who have infertility due to low ovarian reserve or in combination with hormonal treatment. The difference and novelty of our method of ovarian rejuvenation was in obtaining a higher platelet concentration (about 1×10^6 μl), which allowed us to achieve a long-lasting result, after a single procedure, within 12 months. Additionally, for the first time, we proposed and performed a laparoscopically assisted technique for administering PRP into the ovary. The limitation of our study was an impossibility to predict who would benefit from the injection of PRP into the ovaries before treatment. It would require invasive and inhumane investigation including ovarian tissue sampling.

In contrast, the procedure of ovarian rejuvenation itself is safe and uncomplicated in experienced hands. There is excellent potential for further research into the restoration of the ovarian reserve using autologous stem cells, including clinical investigations. We see great promise in the future study of the effects of PRP and stem cells as immunomodulatory and anti-inflammatory agents not only with laboratory animals [15, 16] but for increasing the quality and longevity of human life. Recently, SVF, which contains ADSCs, has gained widespread use in regenerative medicine, as well as for many diseases, including immune disorders, tissue degeneration, and ischemic conditions. We want to provide not enzymatic but mechanical production of SVF, which was determined by Tonnard et al. [17]. An injectable product called "nano-fat" was obtained by the emulsification and filtration of lipoaspirate. ADSCs are perhaps the easiest to get of all the different types of MSCs compared with that which is extracted from the bone marrow [18, 19]. However, care must be taken not to harm the patients. We are currently in the process of designing the methodology. We will carry out the protocol of injections using autologous stromal vascular fraction (SVF) from fat, combined with PRP, into women who did not get results after PRP treatment alone.

Authors' Contribution Both authors contributed equally to this work.

Compliance with Ethical Standards

Competing Interests The authors both hold the UA patent for the process and treatment using PRP injections into the ovaries.

Abbreviation PRP, Platelet-rich autologous plasma; FSH, Follicle-stimulating hormone; LH, Luteinizing hormone; AMH, Anti-Müllerian hormone; TNF, Tumor necrosis factor; TGF, Transforming growth factor; BMP, Bone morphogenic protein; FSHR, Follicle-stimulating hormone receptor; IGF, Insulin-like growth factor; POF, Premature ovarian failure; GF, Growth factor; PDGF, Platelet-derived growth factor; PDAF, Platelet-derived angiogenesis factor; PF, Platelet factor; EGF, Epidermal growth factor; ECGF, Epithelial cell grow factor; VEGF, Vascular endothelial cell growth factor; bFGF, Fibroblast growth factor; GSC, Germline stem cell; IVF, In vitro fertilization; hCG, Human chorionic gonadotropin; SVF, Stromal vascular fraction; MSC, Mesenchymal stem/stromal cell; ADSC, Adipose-derived stem/stromal cell

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