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Management of Poor Responders Patients-Case Series on Platelet Rich Plasma after Intraovarian Injection

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Abstract

Background: Reports on clinical uses of platelet-rich plasma (PRP) have dramatically increased in the last decade. Indications for PRP therapy range from muscle and skeletal injuries to hair re-growth. More recently evidences have shown its positive effects in promoting endometrial and follicular growth and gestation in assisted reproduction cycles. We discuss the alleged role of PRP on endometrial receptivity and ovaries rejuvenation with a brief history of its applications in research and clinical therapies.

Methods: This report presents the cases of 3 womens aged 34,41,41 who have experienced premature menopause or POF. Having rejected oocyte donation, they opted for intraovarian injection of autologous platelet-rich plasma with the aim to rejuvenate the ovarian tissue and enable the employment of her own gametes through in-vitro fertilization. Weeks following the autologous platelet-rich plasma treatment, a significant reduction in the patient's follicle-stimulating hormone (FSH) levels was noted.

Discussion: A natural in-vitro fertilization cycle and natural conception led to a biochemical pregnancy. This is a report of a successful autologous platelet-rich plasma application leading to pregnancy in menopause or POF. This report has a unique contribution to the medical knowledge and challenges current practice in the context of infertility.

Conclusion: Despite its widespread uses in medicine, the mechanisms through which PRP exerts its regenerative effects are only postulated, not based on scientific data.

This cases report cannot serve as the answer to all the questions raised. It does, however, set the basis for further research, data, and evidence regarding PRP implementation in clinical practice to corroborate present findings in the clinical scenario.

Keywords: Premature menopause; Autologous platelet rich plasma; Pregnancy; Rejuvenation; Menstrual restoration

Abbreviations: FSH: Follicle-Stimulating Hormone; GFs: Growth Factor; ADP: Adenoside Diphosphate; ATPS: Adenoside Triphosphate; PRP: Platelet-Rich Plasma; FGF: Fibroblast Growth Factor; EGF: Epidermal Growth Factor; TGF-B: Transforming Growth Factor Beta; P-PRP: Pure Platelet-Rich Plasma; L-PRP: Leucocyte-and Platelet-Rich Plasma; P-PRF: Pure Platelet-Rich Fibrin; L-PRF: Leucocyte and Plateletrich Fibrin; CG: Calcium Gluconate;

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ART: Assisted Reproduction Technologies; ICSI: Intracytoplasmic Sperm Injection; PRG: Intracytoplasmic Sperm Injection; OSCs: Ovarian Stem Cells; GSCc: Germline Stem Cells; SDF-1: Stromal Cell Derived Factor 1; HGF: Hepatocyte Growth Factor; TGF-B1: Transforming Growth Factor Beta Isoform 1; HMGB1: High-Mobility Group Protein B1; HCG: Human Chorionic Gonadotrophin

Introduction

Platelet-rich plasma (PRP) is described as the volume of plasma that has a platelet count above baseline^ [1]. Platelets are small, anucleated cell fragments (2 to 3 μm in diameter) released from megakaryocytes found in the bone marrow. They contain numerous proteins, several growth factors (GFs) [2], and cytokines stored in cytoplasmic granules. The physiological actions of some of the proteins have been studied, including GFs, peptide hormones, and chemoattractants for macrophages, neutrophils, stem cells, and several hundred other proteins, such as fibrinogen and fibrin. In addition, there are proteins with antibacterial and fungicidal actions. The dense granules in platelets contain adenoside diphosphate (ADP), adenoside triphosphate (ATP), calcium ions, histamine, serotonin, and dopamine, which represent important factors for tissue homeostasis.

However, PRP also includes numerous soluble mediators which orchestrate complex immune responses and tissue regeneration. Closely associated with inflammatory signaling, PRP predominates in tissue regeneration and orchestrates a regulatory interplay of cellular migration, extracellular matrix remodeling, cell proliferation, apoptosis, differentiation, and angiogenesis in response to widespread cell damage. Following trauma or local ischemia as with myocardial infarction or stroke, platelets are among the first cells to arrive and, following activation, emit a multitude of biologically active mediators to rectify the tissue trauma [3-5]. Notably, the human ovary is covered by an epithelial monolayer which sustains cyclic 'injury' and local tissue repair with each ovulation.

Platelet-rich plasma (PRP) is a current trend surfacing in the latest published literature. Studies employing animal models in the reproductive context have reported that PRP enhances the development of primordial and primary preantral follicles. Furthermore, PRP has proven efficacious in preventing possible ischemia following ovarian injury. It is already evident that the employment of PRP to target various issues regarding the reproductive system could be beneficial [6-11]. PRP institutes an autologous and highly concentrated solution of plasma, which is prepared from the patient's own blood and contains a concentrated source of growth factors, namely insulinlike growth factor-1 and 2 (IGF-1, IGF-2), fibroblast growth factor (FGF), epidermal growth factor (EGF), transforming growth factor beta (TGF-b), hormones, and cytokines [12]. Considering the active factors involved, along with their potent therapeutic nature, it is not unreasonable to hypothesize that PRP treatment may assist in tissue regeneration, the enhancement of anabolic signalling pathways, cell differentiation and proliferation, angiogenesis initiation, and control [13-16].

After the first round of GFs release at activation, the continued exocytosis maintains GF levels three- to five times higher as compared to baseline values [1]. PRP contains a member of the TGF-b superfamily, growth differentiation factor 9 (GDF-9). The gdf-9 gene expression is regarded as a biomarker of oocyte maturation potential, and its mutations have been linked to premature ovarian failure [17-20]. This finding may constitute yet another piece in the completion of the puzzle regarding the delineation of the therapeutic potential behind PRP.

The possibility of PRP improving the ovarian microenvironment–and even interacting with putative ovarian germline stem cells (GSCs)–warrants serious consideration.

PRP preparation methods and classification

Different techniques for PRP preparation can be found in literature. Each preparation method is intended to create an end product with a particular bio action, and consequently, with a specific clinical application. Thus, PRP is not the only blood derivate containing plasma and high concentrations of platelets. According to Dohan Ehrenfest and colleagues [3], platelet concentrates can be classified in four categories, depending on their leucocyte and fibrin content: pure platelet-rich plasma (P-PRP), leucocyte- and platelet-rich plasma (L-PRP), pure platelet-rich fibrin (P-PRF), and leucocyte and plateletrich fibrin (L-PRF). In each category, the concentrate can be produced by different processes.

Most of the described PRP preparation methods involve similar procedures, such as blood collection in presence of an anticoagulant and immediate centrifugation. The short, mild-spin centrifugation aims to separate the whole blood into three layers: the supernatant corresponding to the cellular plasma, the intermediate "buffy coat" containing the concentrated platelets and last, the bottom pellet rich in red blood cells. After the first centrifugation, a second faster and more prolonged spin may follow, to further isolate the buffy coat. Finally, at administration on the

target site, an activating factor, such as thrombin may be added to the final platelet concentrate to promote platelet degranulation and exocytosis of the factors stored in the cytoplasmic granules. Total platelet count from one patient to another can vary and the overall goal is to achieve a concentrating factor of two to three times in whole blood for any given patient [21].

Methods

Sample preparation

Approximately, 20mL blood was collected from each participant by peripheral venipuncture using a 21 G butterfly catheter affixed via vacutainer to negative pressure receiving tubes (Regen Lab; Mont-sur-Lausanne, Switzerland). Samples were immediately labeled and placed in room-temperature centrifuge set to 1500g 5min [22].

After centrifugation, the blood was fractionated; red blood cells were trapped under the gel while lower density components stratified above the surface of the separator gel. Less than 5mL of supernatant (corresponding to relatively platelet-poor plasma fraction) was next aspirated off the top of each column before recapping the vial for gentle tube inversion/resuspension.

PRP activation and intraovarian injection

PRP activation was achieved with calcium gluconate (CG) similar to previously published methods [22,23]. In brief, syringes were used to divide activated PRP samples into two equal portions and maintained at room temperature, then attached to a 35cm single lumen 21 G needle assemblies (kitazato). This apparatus was modified for office PRP administration by bypassing the falcon tube collection port to allow direct injection into ovarian stroma under transvaginal ultrasound guidance. The ovaries were aligned with the needle guide to avoid itraumatizing vascular or other structures; the needle was quickly advanced without rotation deep into the central ovary.

Once tip placement was confirmed, the activated PRP sample was slowly introduced as the needle was retracted across previously traversed ovarian cortex. The final 1 mL of sample was deposited just under the ovarian capsule as the needle cleared the ovary.

After activated PRP injection was completed bilaterally, careful ultrasound assessment of the pelvis was performed to assure vascular integrity and absence of free pelvic fluid. Sedation or anesthesia was necessary for any ovarian PRP injection; for all study patients this was completed in less than seven minutes. Following the procedure, each patient was asked to remain supine and rest for 15min; vital signs were rechecked before home discharge.

Clinical applications of PRP

In regenerative medicine research, multipotent Mesenchymal stem cells obtained from deciduous teeth or umbilical cord were successfully maintained in culture and retained their differentiation capacity, when xenogeneic fetal calf serum was replaced by autologous PRP [24,25]. These reports represent important steps towards the generation of clinical grade stem cell lineages for human use, free from the presence of animal- derived products during isolation and culture.

PRP and human reproduction

The first trial on the use of PRP in human reproduction technologies was reported by a Chinese group [6] to improve endometrial thickness in patients undergoing IVF treatment. Since the first IVF attempts in the mid-1970s, researchers are aware of the important role played by the endometrium, not only by the embryo itself, in achieving a pregnancy. After nearly four decades of research and clinical trials, Assisted Reproduction Technologies (ART) have significantly improved pregnancy and birth rates, as new methodologies allowed embryonic growth to the blastocyst stage and genetic screening helped to choose “the best embryo “ for transfer.

In the field of fertility preservation for women with cancer, a recent study demonstrated that in vitro follicular growth from primordial or primary to preantral stage is enhanced by the addition of PRP to the culture medium. Isolated primordial or primary follicles from fresh or cryopreserved ovarian tissue cultured for 10 days in presence of PRP showed a significant greater growth rate and cell viability than follicles cultured in medium supplemented with fetal bovine serum. Also, the PRP in auto grafts of cryopreserved human ovarian tissue may have contributed to the successful pregnancy and birth after the first stimulation cycle in an oophorectomized patient, with undetectable level of AMH. These last reports emphasize the putative role of PRP growth factors on cell proliferation and neoangiogenesis promoting follicular survival and development [8,26].

Assessment of PRP effectiveness and interval to IVF

All patients underwent periodic testing for serum AMH, estradiol (E2) and FSH at approximately two-week intervals after ovarian PRP. This study was initially

configured to enroll menopausal or peri-menopausal patients where serum FSH and E2 could be measured randomly, without respect to cycle day. This audit approach required recalibration when some women who were acyclic at study entry, began to menstruate subsequent to ovarian PRP. For such patients, instructions were modified to obtain laboratory testing on cycle day 2 or 3 (for FSH and E2) and then two weeks thereafter. This preserved the general cadence of twice monthly assessments for all study participants. As published data on ovarian reserve marker responsiveness following PRP dosing do not yet exist, post-treatment target thresholds for AMH, E2 or FSH could not be referenced. Nevertheless, when improvements in ovarian reserve (i.e. increased AMH or decreased FSH) were measured on two consecutive tests, patients were advised to commence IVF promptly.

Statistical analysis

Patient data were analyzed with Numbers version 4.3.1 (Apple Inc., Cupertino, CA). For normally distributed data (e.g. patient age), mean and standard deviation were used to describe data location and dispersion. Comparisons across means were evaluated by paired two-tailed Student's t-test. By default, confidence level was set at 95% for all analysis.

Results

In this case report, we describe a singular treatment of a prematurely menopause patient with the intravorian injection of autologous PRP. The motivation behind this approach was to value the hypothesis that PRP implementation could contribute towards partially restoring ovarian function in prematurely menopausal patients.

Case report I

Our patient, aged 34, was diagnosed as being prematurely menopausal at the age of 31, following the absence of menstruation for 10 months. During the first appointentment a detailed reproductive examination was performed, including an ultrasound and a hormonal profile assessment. Prior to entering in menopause the patient reported 2 years of failing to achieve a natural conception. Unexplained infertility for the couple was diagnosed following an initial infertility investigation.

The patient's overall medical record was free of any medical complications. The FSH levels were recorded at 142 mIU/mL , LH mIU/mL and estradiol was 5 pg/mL AMH was 0.8 ng.

As protocol dictates, initially the patient was recommended the option of oocyte donation. This option was rejected and the couple expressed their desire to pursue alternative approaches. The current literature cements the therapeutic nature of PRP for various systems, including the reproductive system.

In vitro studies coupled with the numerous registered clinical PRP trials, prompted us to suggest PRP treatment, especially on the grounds that it involves an autologous, minimally invasive, in-house and complication free approach.

Furthermore, PRP treatment has provided favorable results in our clinic for poor responder patients and patients with chronic endometritis. Following thorough consultation, the patient opted for the PRP approach. The patient provided informed consent prior to participating in the study. The study was conducted in accordance with the Declaration of Helsinki and the protocol was approved by the ethics board of Iaso Hospital
The RegenACR®-C Kit (Regen Laboratory, Le Mont-sur-Lausanne, Switzerland) was employed to prepare the PRP, which was subsequently injected into the ovaries. The volume of peripheral blood required to prepare 10 mL of PRP for administration was 60 mL. The initial concentration of platelets in the peripheral blood sample was 250,000/μL, while the prepared PRP presented with a concentration of 900,000/μL. According to our protocol, the prepared PRP can be stored at a temperature of 4 °C for a maximum of 1 h. This allows an extent of flexibility regarding the timing of administration that may be required in the clinical setting of a large IVF program. Regarding this case, immediate administration of PRP following preparation was possible and hence we proceeded as such. A volume of approximately 5 mL per ovary was employed for the intraovarian injection. As menopausal women present with ovaries of reduced volume, part of the prepared PRP ultimately resulted in the peritoneal area. The essential parts of the technique consisted of a nonsurgical, transvaginal, ultrasound-guided, multifocal, intramedullary injection, and diffusion in the subcortical layers. The injection included multiple sites and, therefore, three punctures were performed per ovary. A 21-gauge needle was employed during the procedure.

Restoration of the menstrual cycle was reported 3 weeks following PRP application. The recorded FSH levels were 9 mIU/mL and the AMH levels were 0,8ng/mL. Employing a natural cycle and natural conception was decided. The patient's follicular growth was assessed via ultrasonography. Five months after no conception was achieved and the FSH levels returned at 60 mIU/mL. It

was decided another PRP treatment. After three months with the FSH levels were 8.9 mIU/mL was decided this time the approach of an Ivf cycle, using of goal 300 iu, Luveris 75mg, not included the use of an antagonist.

The patient was subjected to an oocyte collection three months after PRP administration. The patient's follicular growth was assessed via ultra sonography and hormonals levels were monitored on days 6,8,10 of stimulation cycle. The follicular diameter were recorded at 12mm, 11mm, 10mm on day 6, 14.5mm, 12.5 ,11.5 on day 8, 17.5,17, 17 on day 10 respectively. Oestradiol (E2) levels were similarly evaluated and recorded at 160pg/mL 270pg/mL, 580 pg/mL respectively.

Progesterone (PRG) levels were also evaluated on day 10 and recorded at 1,2ng/mL. Following the administration of human chorionic gonadotrophin (hcg) on day 10, oocyte retrieval was enabled 36h later. Two oocytes was retrieved, one mature was inseminated employing intracytoplasmic sperm injection (ICSI). A normally fertilized zygote with two pronuclei led to a six-cell cleavage stage embryo of average grade three qualities, according to the morphology grading criteria developed by Veeck and colleagues [2]. Embryo transfer was performed using ultrasound guidance and a 23 cm soft-pass embryo replacement catheter with an echogenic tip. The luteal support scheme included one tablet of cyclacur (estradiol and levonorgestrel), every 8 h per os (oral administration of medication), and vaginally dosed progestin (200 mg) three times per day. Following the embryo transfer, a biochemical pregnancy ensued and was confirmed by a positive human chorionic gonadotropin (hCG) level of 102 mIU/mL. Subsequent hCG measurements were 182 mIU/mL and 800 mIU/mL.

Case report II

Our patient, aged 41, was diagnosed as POF at the age of 37.The patient reported three IVF attempts with PGS. All embryos results aneuploidic. The patient was recommended the option of oocyte donation but this option was rejected by the couple, expressed their desire to pursue PRP treatment. The recorded FSH levels before the PRP treatment was FSH 22 mIU/mL and AMH was 0,06ng/ml.

The patient provided informed consent prior to participating in the study. The study was conducted in accordance with the Declaration of Helsinki and the protocol was approved by the ethics board of Iaso Maternity Hospital. The RegenACR®-C Kit (Regen Laboratory, Le Mont-sur-Lausanne, Switzerland) was employed to prepare the PRP, which was subsequently injected into the ovaries. The volume of peripheral blood required to prepare 10 mL of PRP for administration was 60 mL. The initial concentration of platelets in the peripheral blood sample was 248,000/μL, while the prepared PRP presented with a concentration of 930,000/μL. According to our protocol, the prepared PRP can be stored at a temperature of 4°C for a maximum of 1 h. This allows an extent of flexibility regarding the timing of administration that may be required in the clinical setting of a large IVF program. Regarding this case, immediate administration of PRP following preparation was possible and hence we proceeded as such. A volume of approximately 5 mL per ovary was employed for the intraovarian injection. As menopausal women present with ovaries of reduced volume, part of the prepared PRP ultimately resulted in the peritoneal area. The essential parts of the technique consisted of a nonsurgical, transvaginal, ultrasound-guided, multifocal, intramedullary injection, and diffusion in the subcortical layers. The injection included multiple sites and, therefore, three punctures were performed per ovary. A 21-gauge needle was employed during the procedure.

Employing a natural cycle , not including the use of stimulation drugs or the use of an antagonist, the patient has subjected to an ultrasound and hormonal levels control every month, 30 days after PRP treatment FSH levels were 13.9 mIU/mL and AMH 0.30 ng/ml. Two months after FSH was 12.5 mIU/mL and AMH 0,20ng/ml. After three months naturally conceived pregnancy was occurred. Harmony test was performed at 10 weeks of pregnancy with no result of aneuploidy.

Case report III

Our patient, 41 aged with low level of AMH 0,1ng. Perimenopause and multimiscarriages were diagnosed following an initial investigation. Six miscarriages with abnormal chromosomal aneuploidies were diagnosed in the past. The patient was recommended the option of oocyte donation. The couple rejects this option and expressed their desire to pursue with the PRP treatment. The patient provided informed consent prior to participating in the study. The study was conducted in accordance with the Declaration of Helsinki and the protocol was approved by the ethics board of Iaso Maternity Hospital. The RegenACR®-C Kit (Regen Laboratory, Le Mont-sur-Lausanne, Switzerland) was employed to prepare the PRP, which was subsequently injected into the ovaries. The volume of peripheral blood required to prepare 10 mL of PRP for administration was 60 mL. The initial concentration of platelets in the peripheral blood sample was 232,000/μL, while the prepared PRP presented with a concentration of

788,000/μL. According to our protocol, the prepared PRP can be stored at a temperature of 4°C for a maximum of 1hr. This allows an extent of flexibility regarding the timing of administration that may be required in the clinical setting of a large IVF program. Regarding this case, immediate administration of PRP following preparation was possible and hence we proceeded as such. A volume of approximately 5 mL per ovary was employed for the intraovarian injection. As menopausal women present with ovaries of reduced volume, part of the prepared PRP ultimately resulted in the peritoneal area. The essential parts of the technique consisted of a nonsurgical, transvaginal, ultrasound-guided, multifocal, intramedullary injection, and diffusion in the subcortical layers. The injection included multiple sites and, therefore, three punctures were performed per ovary. A 21-gauge needle was employed during the procedure.

Twenty days after PRP treatment in the 2 day of menstrual cycle the FSH level was 9 mIU/mL and AMH 0,23ng/ml. On the second month after the PRP treatment a natural conceive pregnancy was established. Harmony test was performed at 10 weeks of pregnancy with no result of aneuploidy.

Discussion

PRP is a part of autologous plasma that has platelets higher than baseline concentration. Platelets store various growth factors and cytokines in their cytoplasmic granules that undergo exocytosis in the presence of activating factors such as collagen of extracellular matrix [27]. PRP is prepared from peripheral blood without risk of viral infection and immunological reactions.

Based on leukocyte and fibrin content, there are four categories of PRP; pure platelet-rich plasma (P-PRP), leukocyte- and platelet-rich plasma (L- PRP), pure platelet-rich fibrin (P-PRF) and leukocyte and platelet-rich fibrin (L-PRF). PRP has been used in gynecological disorders including Asherman syndrome management symptomatic vaginal mesh exposure, wound healing after cesarean section, treatment of thin endometrium following hormone therapy for embryo transfer and premature ovarian failure [29-32]. PRP has a role in tissue regeneration by way of clinically relevant but still poorly understood processes controlling apoptosis, cell survival, and tissue repair or rescue.

The activated PRP substrate explored in the current study is known to be comprised of cytokines, chemokines, and growth factors including stromal cell derived factor 1 (SDF-1) and hepatocyte growth factor (HGF) which control recruitment, proliferation, and activation of fibroblasts, neutrophils, monocytes and other cells central to wound healing. Within the human ovary, PRP would also be expected to deliver mediators regulating angiogenesis and tissue perfusion which might serve an independent or accessory role in improving oocyte competency. Platelet alpha granules are known to release transforming growth factor beta isoform 1 (TGF-b1), a prime regulator of cell proliferation, angiogenesis, and extracellular matrix deposition [33-36]. The balance between apoptosis and cell survival is thus governed by PRP components, which release proapoptotic (Fas- L, CD40L, TRAIL, TWEAK, and LIGHT) and anti-apoptotic (HGF, SDF-1, serotonin, adenosine di-phosphate, and sphingo- sine-1-phosphate) signals. High-mobility group protein B1 (HMGB1) is a 'danger signal' that is exported to the cell surface by platelets upon activation, modulating apoptosis as well as autophagy as a function of local redox status [37].

Given the highly angiogenic structure of the ovary and the critical role of various platelet-derived factors in vascular activation and stabilization, PRP application is proposed to have enriched the dysfunctional and prematurely menopausal ovarian tissue of our patient with essential factors for neoangiogenesis. In addition, the detection of ovarian stem cells (OSCs) or germ line stem cells (GSCs), and embryonic-like stem cells (VSEL) in human ovarian surface epithelium-even of post-menopausal and POF women and their ability to differentiate into oocytes under certain conditions create new data for the origin of PRP-derived follicles. The significantly decreased levels of FSH following PRP treatment constituted an improvement in regard to the overall reproductive potential of our patient. Thus, this interesting outcome could present an indicative marker of the therapeutic nature of PRP application. On another note, the authors refrain from any statements regarding the slightly increased AMH levels, since the interpretation of the fluctuating AMH levels-even during the menstrual cycle-is often described as conflicting in the literature [38-41].

The changes observed in ovarian reserve after PRP treatment here are difficult to interpret. Fundamentally, any alteration in ovary function associated with insertion of this wide array of cell signals could have occurred for at least two reasons. One scenario is that the oocytes we recovered within several weeks of ovarian PRP administration were actually there all along, always present but latent, and simply awakened with the arrival of numerous cellular growth factors generated by a small bolus of autologous platelets in tandem with standard gonadotropins. An alternate possibility is that growth signals associated with PRP established communication with uncommitted ovarian stem cells, and provided the

proper milieu required to induce differentiation to develop de novo oocytes.

In any case, considerable additional investigation is planned to explore and disentangle these issues, aiming to provide a better understanding of how the ovary behaves following PRP dosing. This study is limited by several factors, including small sample size, the absence of a placebo control group Study patients were selected because they were considered a very poor prognosis, as all had been classified as refractory cases and referred for donor oocyte IVF elsewhere. Against this back- ground, it was proposed that the tissue repair processes associated with PRP [42] might generalize to the ovary, and our preliminary findings appear to validate this hypothesis.

It may be important to note that no adverse side effects were reported following treatment. In fact, the patient described the PRP treatment as a 'psychological boost' towards her perception of her reproductive status. PRP may enable the "hope" of a positive outcome in premature menopause, which is commonly accepted as a final scenario.

If further proof of the application of PRP is presented in the future, validated by the anticipated large clinical trials, will it be safe to extrapolate that it could become an option for prematurely menopausal women aiming to achieve a pregnancy?

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