Title: The use of autologous platelet-rich plasma (PRP) *versus* no intervention in women with low ovarian reserve undergoing fertility treatment: a non-randomized interventional study

Running title: PRP injections improve ovarian reserve markers

Abstract

Purpose

To investigate the impact of a 3-month course of intracortical injections of autologous platelet-rich plasma (PRP) upon ovarian reserve markers *versus* no intervention in women with low ovarian reserve prior to undergoing assisted reproductive technology (ART).

Methods

Prospective controlled, non-randomized comparative study conducted in a private fertility clinic, Venezuela. Women with abnormal ovarian reserve markers (FSH, AMH and AFC) who declined oocyte donation were allocated to one of the following groups according to patient choice: monthly intracortical ovarian PRP injections for three cycles, or no intervention. Primary outcomes were the change in FSH, AMH and AFC pre- and post-treatment. Secondary outcomes included the number of oocytes collected and fertilized; biochemical/clinical pregnancy rates; and miscarriage and live birth rates.

Results

Eighty-three women were included, of which 46 received PRP treatment and 37 underwent no intervention. Overall median age was 41 years (IQR 39-44). There were no demographic differences between the study groups. At the three-month follow-up, women treated with PRP experienced a significant improvement in FSH, AMH and AFC, whereas there was no change in the control group. Furthermore, overall the rates of biochemical (26.1% *versus* 5.4%, P=0.02) and clinical pregnancy

(23.9% *versus* 5.4%, *P*=0.03) were higher in the PRP group, while there was no difference in the rates of first trimester miscarriage and live birth between groups.

Conclusion

PRP injections are effective and safe to improve markers of low ovarian reserve prior to ART, although further evidence is required to evaluate the impact of PRP on pregnancy outcomes.

Key words

Platelet-rich plasma; ovarian reserve; assisted reproductive techniques; pregnancy outcome

Introduction

Female age remains by far the principal limiting factor of success in both spontaneous conception and assisted reproductive technology (ART), largely due to a loss of ovarian follicle reserve and oocyte quality as women become older [1]. Indeed, the total number of oocytes in the developing female fetus peaks at 6 million in the second trimester of gestation and steadily declines thereafter [2]. At birth, both ovaries contain 1-2 million oocytes, and more than half will undergo atresia before a woman reaches puberty [3, 4]. The rate of follicle degeneration increases after the age of 37 years and only 1000 oocytes, on average, are present at the time of the menopause [1, 5]. Alongside a reduction in numbers, aging oocytes are also more prone to errors in DNA synthesis and cell division, resulting in increased rates of aneuploidy and congenital defects in the progeny of older women [6].

There is no known effective treatment to prevent, delay or reverse ovarian senescence. Environmental factors such as cigarette smoking [7, 8], dietary habits [9] and exposure to chemo and radiotherapy [10] are known to irreversibly reduce oocyte numbers and quality, mainly via the excessive production of reactive oxygen species (ROS) [11]. Antioxidant dietary supplements containing vitamins C and E [12, 13], melatonin [14], dehydroepiandosterone (DHEA) and coenzyme Q10 [15, 16] have thus been used in reproductive medicine to reduce oxidative stress and improve ovarian reserve, but evidence attesting to their overall effectiveness remains sparse and meta-analyses have been inconclusive [17, 18].

Over the past two decades, regenerative medicine has benefited from significant advances in the field of tissue engineering [19]. The use of platelets in particular has been shown promising due to their role in triggering cell proliferation and tissue differentiation [20]. When activated by external stimuli such as haemorrhage and tissue damage, platelets release multiple bioactive molecules and growth factors that induce clotting, inflammation, neovascularization and local tissue repair [20, 21]. The healing properties attributed to platelet function have led to the use of platelet-rich plasma (PRP), a concentrate derived from centrifuged whole blood with platelet concentrations up to seven times higher than circulating serum, in regenerative medicine [22]. It has been postulated that the heightened regenerative properties of PRP may be explained by higher concentrations of growth factors such as transforming growth factor-ß, insulin-like growth factors 1 and 2 (IGF-1 and -2), vascular endothelial

growth factor (VEGF), epidermal growth factor (EGF), basic fibroblast growth factor and hepatocyte growth factor (HGF) [20, 22, 23].

In vitro and clinical studies have investigated the applicability of PRP as a therapeutic agent in nerve injuries [24], myocardial infarction [25], cosmetic surgery [26, 27] and eye disease [28]. Furthermore, PRP has been increasingly utilized in sports medicine to treat ligament and tendon lesions due to its association with shortened recovery times and improved functional outcomes, but there is a paucity of good-quality scientific evidence demonstrating its effectiveness [29, 30].

Very few studies have investigated the potential applicability of PRP in ovarian tissue regeneration [31]. Bakacak et al demonstrated a significant effect of PRP in preventing ischemia and reperfusion damage in rats following bilateral adnexal torsion and surgical detorsion, mainly through an increase in VEGF [32]. Other small case series evaluating the role of PRP in women with a thin endometrium [33], recurrent implantation failure [34] and poor response to controlled ovarian stimulation [35] have since been published with encouraging results, but there have been no controlled clinical studies investigating the effectiveness of PRP in women with low ovarian reserve. It is thought that PRP may be beneficial in delaying follicle atresia and oocyte degeneration, but conclusive evidence is lacking.

This study aimed to evaluate the effectiveness of PRP compared with no intervention in women with known low ovarian reserve prior to undergoing ART.

Materials and methods

Study Design

This prospective non-randomized comparative pilot study was conducted between February 2015 and February 2018 in a private fertility clinic in Caracas, Venezuela. We included women who fulfilled the following criteria prior to undergoing ART: (i) female age 38 years old and above, (ii) baseline follicle-stimulating hormone (FSH, day 3 of the menstrual cycle) > 12 mIU/mL, (iii) anti-müllerian hormone (AMH) <0.8 ng/mL and (iv) normal uterine cavity as demonstrated by recent hysteroscopy. The following exclusion criteria were applied: (i) previous history of pelvic inflammatory disease, (ii) known clinical/biochemical hyperandrogenism or polycystic ovaries, (iii) tubal factor infertility, (iv)

endometriosis, (v) known platelet or thromboxane synthesis disorder and (vi) known severe male factor infertility.

Ethical Approval

This study was designed, conducted and reported in accordance with the principles of Good Clinical Practice guidance and with the 1964 Helsinki declaration and its later amendments. Prospective ethical approval was granted by a local Institutional Review Board (IRB) and the Venezuelan Health Ministry (IRB reference number #0940), and written consent was obtained from all participants.

Patient allocation

Women planning to undergo fertility treatment (timed intercourse, IUI or IVF/ICSI), who fulfilled the inclusion criteria, were initially informed about the trial and allocated to one of the following groups according to patient choice: ovarian injection with autologous PRP or no intervention. Baseline antral follicle count (AFC) on transvaginal ultrasound and serum levels of FSH and AMH were obtained from all participants on day 3 of menstrual cycle 1.

Ovarian PRP injection

Participants who opted for PRP injections received treatment once between days 7 to 9 of the menstrual cycle for three consecutive cycles (cycles 1, 2 and 3). The decision to undertake three treatment cycles derived from the knowledge that antral follicle development takes approximately 90 to 120 days. We postulated that repeated platelet stimulation would maximise the number of growing follicles exposed to the intervention.

Platelet-rich plasma was initially obtained from whole blood collected on the day of injection. A total of 5 blood collection tubes containing sodium citrate 3.8% were filled with 4.5 ml of blood each and centrifuged at 270 g for 10 minutes. Following centrifugation, 100 μL of the platelet-rich supernatant were transferred from each of 4 of the original blood tubes and mixed with 0.1 ml of 10% Calcium Chloride. The blood in the remaining fifth tube was not mixed with Calcium Chloride to allow for quantification of the total number of platelets in the sample.

On the day of blood collection (i.e. day 7, 8 or 9 of the cycle), 200 μL of PRP were injected into the cortex of each ovary using a single lumen aspiration needle (Cook Medical, USA) under transvaginal ultrasound guidance and sedation. Each ovary was punctured once only, with the single lumen needle being inserted into the ovarian cortex superficially, and a total of 200 μL PRP injected into the subcortical area of the ovary.

FSH, AMH and AFC measurements were repeated on day 3 of the cycle following the last round of treatment (i.e. cycle 4) and compared with pre-treatment results (i.e. cycle 1). Following the completion of treatment with PRP, participants were advised to undergo IVF/ICSI, IUI or timed intercourse as soon as the next menstrual cycle started.

Controls

Women who volunteered to participate in the study but declined to receive treatment with PRP were allocated to the control group, in whom no intervention was carried out apart from measuring ovarian reserve parameters in cycles 1 and 4.

Conception

Women in both groups were followed up for a total of 12 months while undergoing subsequent ART. Details of fertility treatment following participation in the study were gathered.

IVF and embryo transfer were carried out following a short GnRH-antagonist protocol. Specifically, all patients received recombinant FSH (rFSH) from day 3 of the cycle, with doses varying from 225 to 300 IU, in addition to 75 to 150 IU of human menopausal gonadotrophin (hMG) for 10-12 days, depending on age, BMI, basal FSH and antral follicle count. Furthermore, cetrorelix acetate (Cetrotide®, Merck KGaA, Dermstadt, Germany) was administered subcutaneously at a dose of 0.25 mg once daily starting from day 5-7 of the cycle, according to follicle size on ultrasound, to prevent premature ovulation, until the day of hCG injection. As soon as one or more follicles measuring ≥17 mm were identified on transvaginal ultrasound, a fixed dose of recombinant human chorionic gonadotrophin (rhCG) 500 mcg (Ovidrel®, Merck KGaA, Dermstadt, Germany) or 5000 IU (Pregnyl®, MSD, Brussels, Belgium) was administered subcutaneously to induce oocyte maturation. Oocyte

collection was performed 36-37 hours post-rhCG, and 1 or 2 embryos were transferred between days 2 to 5 depending on patient response and embryo quality.

The local IUI protocol entailed the administration of 100 to 150 IU of rFSH and 75 IU of hMG from day 3 of the cycle for 10-12 days. Once one or more follicles measuring ≥17 mm were observed on ultrasound, a fixed dose of rhCG 250 mcg (Ovidrel®, Merck KGaA, Dermstadt, Germany) or 5000 IU (Pregnyl®, MSD, Brussels, Belgium) was used to induce oocyte maturation. IUI was carried out 42 hours after rhCG injection, followed by a second insemination 24 hours later.

All participants undergoing timed intercourse received 75 IU of rFSH for 5-10 days. Where no follicles measuring ≥17 mm were visualized with transvaginal ultrasound following rFSH injection, a further 75 IU of HMG for 5-10 days were administered. As soon as one of more follicles measuring ≥17 mm were identified, ovulation was triggered with rHCG 250 mcg (Ovidrel®, Merck KGaA, Dermstadt, Germany) or 5000 IU (Pregnyl®, MSD, Brussels, Belgium), followed by sexual intercourse over a period of three days.

Outcome variables

The primary outcome variables were the AFC (defined as all follicles measuring 3-8 mm) on repeat transvaginal ultrasound and the serum levels of FSH and AMH as a measure of ovarian reserve. A single operator (CN) performed the ultrasound assessment of AFC pre- and post-intervention (i.e. in cycles 1 and 4, respectively), using the same equipment. All samples containing baseline ovarian reserve markers prior to treatment were frozen and analysed in cycle 4 (i.e. after completion of the three treatment cycles in the PRP group) using the same assay to avoid clinician, operator and assessor bias. AMH quantification was performed using the Ultra-Sensitive AMH/MIS ELISA assay AL-105-I (AnshLabs, Texas, USA) and .FSH quantification was carried out with a Reactiva Search FSH kit (One Global Search, Florida, USA).

Secondary outcomes included number of oocytes collected and fertilization rates during IVF/ICSI; rates of biochemical (diagnosed by the detection of beta hCG in serum [>5 mIU/mL] or urine), clinical (diagnosed by ultrasonographic visualization of ≥1 gestational sac) and ongoing (12 weeks' gestation

and above) pregnancy per participant; and rates of first trimester miscarriage (<12 completed weeks' gestation) and live birth (≥24 completed weeks' gestation) per participant.

Data collection and statistical analysis

Data were recorded prospectively on all participants. Details of pregnancy outcomes were obtained from hospital obstetric records.

Continuous variables were assessed for normality with the Shapiro-Wilk test, and results were expressed as the median and interquartile range (IQR) or range. A Mann-Whitney U test was used to assess for differences in continuous variables between the two groups, whereas a Wilcoxon Signed-Rank test was used to establish univariate comparisons before and after treatment with PRP or no intervention within the same group. For categorical data, significant differences were identified with a Chi-squared (χ^2) test or Fisher's exact test.

Differences were considered significant where $P<0.05$. The statistical software package SPSS 22.0 (IBM, Chicago, IL) was used for the analyses of all data.

Results

Study Population

Figure 1 depicts details of patient enrolment in the study, allocation, fertility treatment (IVF/ICSI *versus* timed intercourse/IUI) and pregnancy outcomes. A total of 120 women were assessed for eligibility. Of these, 15 declined to participate in the study and 22 were excluded due to the following reasons: severely abnormal semen analysis in the male partner; tubal factor infertility; and successful spontaneous conception prior to undergoing treatment. Of the 83 women included in this comparative analysis, 46 underwent treatment with PRP and 37 were subjected to no intervention.

Demographic characteristics, baseline ovarian reserve markers and ultrasound findings of women included in the analysis were similar among the treatment arms and are shown in Table 1. In particular,

no significant age difference was identified between the study arms (median age overall 41 years, IQR 39-44) (P=0.78). No patients were lost to follow-up.

Ovarian reserve parameters

Women treated with PRP had the most significant improvement in biochemical and ultrasound markers of ovarian reserve compared to the control group (Table 2). Notably, AMH levels were on average 63% higher following PRP (P<0.001) compared with no significant change in the control group (P=0.15). FSH levels dropped by 33% in the group receiving PRP (P<0.001) and remained the same in controls (P=0.23). Finally, there were on average 75% more antral follicles on ultrasound following PRP (P<0.001), while there was no change in controls at the 3-month follow-up (P=0.1).

Cycle characteristics

Of the 40 women who underwent IVF/ICSI following participation in the study (Table 3), those with previous PRP treatment yielded on average more than 1.5x the number of oocytes collected in the control group (P<0.001). The median number of fertilized oocytes and the fertilization rate did not differ between groups (P=0.38 and P=0.51, respectively). Nevertheless, the rate of medium- and top-quality embryos in participants who received PRP treatment was significantly higher than in controls (100% *versus* 55% respectively, P=0.03). There were no differences between groups in the number of embryos transferred (P=0.96) and the day of embryo transfer (P=0.28).

Pregnancy outcomes

An overall comparison of pregnancy outcomes between the two study arms is presented in Table 4. Treatment with PRP was significantly linked with higher biochemical (P=0.02) and clinical pregnancy rates (P=0.03), although the rates of first trimester miscarriage and live birth did not differ between treatment groups. A subgroup analysis according to ART modality (timed intercourse/IUI *versus* IVF/ICSI) did not identify any differences in pregnancy outcomes between those who had previously been treated with PRP and those who had undergone no intervention (Table 5).

Adverse events associated with PRP

There were no significant complications such as allergic reactions, intraabdominal haemorrhage, bowel or bladder injury or infection following treatment with PRP. There were no cases of ovarian hyperstimulation syndrome (OHSS) following IVF/ICSI.

Discussion

This non-randomized controlled pilot study compared the effect of PRP *versus* no intervention upon ovarian reserve parameters in women with low ovarian reserve prior to ART. Our findings showed that a three-month treatment with PRP improved ovarian reserve markers when compared to no intervention. In addition, the use of PRP was associated with a significant increase in biochemical and clinical pregnancy rates. Nevertheless, our data revealed no effect of PRP on the rates of oocyte fertilization in IVF/ICSI, miscarriage and live birth.

The paradigm of inevitable ovarian senescence has long been based on evidence that the female gonads lose their ability to generate new oocytes prior to birth. This longstanding belief postulates that a finite number of oocytes in females of reproductive age are arrested in meiosis I and surrounded by a single layer of squamous pre-granulosa cells forming a primordial follicle [36]. A series of complex bidirectional signalling pathways between the surrounding ovarian tissue and the oocyte involving molecules such as TGF-β, PDGF and IGF-1 lead to the recruitment of some primordial follicles which develop into primary, secondary and antral follicles during the female reproductive years [23, 36-38]. While most growing follicles undergo apoptosis and atresia, ~400 will reach full development and release mature oocytes throughout the course of a woman's reproductive life [39]. Once the pool of primordial follicles is exhausted, folliculogenesis comes to a halt and women enter the menopause, usually after 50 years [40, 41]. When the depletion of primordial follicles occurs earlier than 40 years, a loss of ovarian function ensues, leading to premature ovarian insufficiency (POI), a condition affecting 1% of women [42].

Recent studies have introduced the concept of neo-oogenesis by demonstrating that contrary to previously believed, it is possible to obtain mitotically-active germ cells from healthy adult ovarian tissue in mice and humans [43, 44]. While our data suggest a resurgence of ovarian activity following the injection of PRP in women older than 40 years, the pathways through which concentrated platelets improve ovarian reserve remain unclear. Mechanistic studies to elucidate the biochemical actions of PRP are lacking, but we speculate that the high levels of PDGF, TGF- β, IGF-1/2, VEGF and EGF identified in platelet concentrates [23, 45] are likely to play a significant role in stimulating the development of pre-antral follicles during the three cycles of treatment, leading to an increase in circulating levels of AMH and in the number of antral follicles generated per menstrual cycle.

The decision to undertake three cycles of treatment with PRP was based on the notion that follicle development takes on average 90 to 120 from the time of primordial follicle recruitment to the final stages of antral development, when a follicle either becomes atretric or releases an oocyte at the time of ovulation [40, 41]. Crucially, however, the resurgence of ovarian activity following PRP in this cohort is unlikely to do with increased recruitment of primordial follicles, as it would have taken well over three months for these to become hormone-sensitive [46]. Instead we postulate that PRP is likely to stimulate the development of existing pre-antral follicles or prevent atresia. Moreover, we did not demonstrate a higher fertilisation rate following treatment with PRP in women undergoing IVF/ICSI, suggesting that higher numbers do not necessarily translate into better oocyte quality. Still, a higher proportion of medium- or top-quality embryos were created in the intervention group compared to controls, indicating that there may be a positive effect of PRP on embryo development following fertilisation. Nevertheless, the small number of participants precludes us from drawing a relationship of causality between PRP treatment and embryo quality, and larger randomized controlled trials are required to validate our findings.

This is, to our knowledge, the first prospective trial investigating the impact of PRP on ovarian reserve and pregnancy outcomes in women with known low ovarian reserve. Previous research focused on the use of PRP in reducing ischaemia-reperfusion injury in rat ovaries [32], and the endometrial effects of platelet-derived products in humans. The use of intrauterine G-CSF alone has indeed been trialled in unselected women undergoing IVF, and no significant difference was identified in endometrial thickness following the administration of G-CSF *versus* placebo [47]. A subsequent study

demonstrated a significant increase in endometrial thickness and implantation rates in women with a thin endometrium following intrauterine injection of PRP, although with unclear statistical significance [48]. More recently, a small case series of four patients with low ovarian reserve revealed a significant improvement in ovarian function in those who received intraovarian PRP [49].

There have been no studies on the long-term effects of PRP in older women. It would be useful to elucidate the systemic effect of PRP, particularly on circulating estradiol levels, and to investigate whether there are potential benefits on cardiovascular and bone health. Cohort studies are hence required to ascertain whether there is a long-term rise in estradiol following PRP and its potential impact on hypoestrogenic women.

The prospective nature of our study, involving data collected over 2 years, is its main strength. Women were followed individually from recruitment to the time of ART and, where applicable, detailed pregnancy outcomes were recorded. In addition, the comparative design allowed for a robust assessment of the efficacy of PRP *versus* a control group with similar demographic baseline characteristics.

The main limitation of this study is that it was non-randomized. Women were assigned to the study groups on a voluntary basis after receiving information on the evidence about the use of PRP in sports and regenerative medicine. This may have been a significant source of selection bias, owing to participants of a higher socio-economic status potentially being more likely to request a self-funded intervention than opting to be allocated to the control group. Overall, however, there were no significant differences in baseline characteristics between the study groups. Another limitation of this study is that our numbers are low, and thus likely insufficient to detect potentially relevant effects on pregnancy outcomes including clinical pregnancy rate, miscarriage rate and live birth rate. Adequately powered randomized parallel studies, with long-term follow-up data, are required to evaluate the continuing impact of PRP on ovarian function and live birth rates in order to corroborate the clinical applicability of our findings.

Conclusions

This study revealed that the injection of PRP into human ovaries is safe and improves ovarian reserve markers as measured by antral follicle count and serum levels of AMH and FSH. In addition, treatment with PRP was linked to overall higher rates of biochemical and clinical pregnancy.

Author's roles

P.M contributed to the study design, data analysis and interpretation, and drafted the manuscript. C.N. contributed to the study design and implementation, patient recruitment, PRP administration, data acquisition, and critically revised the manuscript. C.J. contributed to the study design and critically revised the manuscript. K.C. contributed to the study design and critically revised the manuscript. L.C. developed the concept and design of the study and had overall responsibility for trial registration, seeking ethical approval, data collection and interpretation, manuscript drafting and critical revisions. All authors critically revised the article for intellectual content and gave final approval. All authors accept responsibility for the paper as published.

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Conflict of interest

The authors have no competing interests to declare.

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