# Combination of CO2 laser and Platlet Rich Plasma: A Safe Treatment as a Possible New Frontier for Poor Ovarian Responders

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**Abstract:** The ovarian reserve defines the number of antral follicles in the woman's ovaries. Female gametes from the twentieth week of intrauterine life undergo a process of inexorable progressive exhaustion and the follicles present at the birth continue to reduce in number, due to a degenerative phenomena that determine its loss. The decrease in ovarian reserve is associated with a reduced fertility and this matter represents the major challenge in reproductive medicine. The interventions that support the regeneration of gonads represent the new frontier. This study intends to propose a method to induce a regeneration of ovarian response in ovarian tissue of patients with poor response and to optimise the recruitment of pre-existing dormant follicles to improve the ovarian stimulation in vitro fertilization cycle. We enrolled patients of childbearing age with poor ovarian reserve diagnosed with AFC <5-7 follicles and /or altered hormonal levels (FSH> 12 mIU / ml; AMH <0.5-1,1 ng / ml) and / or with at least one IVF cycle that has given failed oocytes recovery. The main scientific and technical innovation of the study is the combination of treatment with CO2 laser (microdissection on both ovaries performed by laparoscopy) and injection of platlet rich plasma on ovarian subcortical tissue under direct vision. DUOSTIM (double stimulation) to start in a month was planned for all enrolled patients. The aim is to demonstrate that the experimental treatment has a safety profile, with no risk for patients health. No patients manifested any adverse reactions and complications. Data on the effect on the fertility restoration are not yet avilable because patients follow up has not yet ended, thus we will publish them in the near future.

**Keywords:** Platlet Rich Plasma, CO2 Laser, Decreased Ovarian Reserve, Poor Responder.

# INTRODUCTION

The ovarian reserve defines the number of antral follicles in the woman's ovaries. Female gametes are formed during fetal life and from the twentieth week of intrauterine, when they are already fully formed, the oocytes begin a process of inexorable progressive exhaustion, undergoing a process of apoptoSsis (cell death). At birth, many primordial follicles are present in a number varying between 700,000 and 1 million (1). Physiologically there is also a progressive numerical reduction of the primordial follicles due to a degenerative phenomena that determine its loss (2).

In adult women, the percentage of reduction of the ovarian reserve is not constant, but it get worse with age. Therefore, at 30 years, the residual follicular patrimony is about 12% of that present at birth. At 40 years, this percentage is reduced to 3% (3).

The decrease in ovarian reserve is associated with a reduced fertility and this matter represents the major challenge in reproductive medicine. The definition of reduced ovarian reserve still remains unclear today and differents conditions exsist: ovarian failure insufficiency (POI), premature ovarian failure (POF) and poor ovarian response (POR).

The European Society of Human Reproduction and Embryology (ESHRE) describes POF as the presence of: amenorrhea for 4 months or more before the age of 40, accompanied by a level of Follicle-stimulating hormone (FSH) > 25 IU/l twice four weeks apart (4).

The Bologna's ESHRE consensus defines women as "poor ovarian responder" in case of at least two of the following three characteristics: (i) advanced age (≥40 years) or any of the risk factors for POR, (ii) a previous poor ovarian response (≤3 oocytes with a protocol of conventional stimulation) and (iii) an abnormal ovarian reserve test (e.g. antral follicle (AFC) <5-7 follicles or AMH <0.5-1.1 ng/ml (5).

The deleterious effects of aging on ovarian reserve and oocyte quality are well known (6). Women with low ovarian reserve generally give inadequate responses and they represent the 9% - 24% of patients seeking pregnancy at specialist In Vitro Fertlization (IVF) centres . The egg donation is a therapeutic option but it involves considerable psychological burdens and meets regulatory restrictions in many countries.

Therefore, the interventions that support the regeneration of gonads represent the new frontier (7). To date, the big limitation is that there is not currently "conventional" therapy that can improve the ovarian response to stimulation in women considered "poor responders" or whose hormonal dosages are close to those of an ovarian reserve in exhaustion (8).

Interest in the use of blood components for non-transfusional use (EunT), for purposes other than those of transfusional support, has rapidly expanded in recent years to various clinical applications in different medical fields.

Growth factors, like PDGF, TGF-1, TGF-2, IGF1, BMP-2, BMP-7, etc, have been used to induce and accelerate the process of healing, for example in the form of PRP (platelet-rich plasma) (9)(10).

The advantage of using the PRP compared to using growth factors purified, lies in providing the complex of growth factors is able to stimulate different types of cells, depending on the graft site and to induce an adequate regenerative response based on key events in tissue repair and regeneration processes.

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Exclusion criteria were oncological or haematological diseases, autoimmune or oncological diseas in treatment with immunotherapy or antiblastic drugs, chronic therapy with antiplatelet and corticosteroids, anemia (Hb <11.5 g / dl) or thrombocytopenia (plt <150,000 / microL), cardiovascular diseases.

The preparation of the PRP was performed at the Transfusion Centre.

The phase of the development of biotherapy consisted in the preparation of PRP through a blood sampling, to obtain as a final product an amount of PRP of 0.3x109 platelets x cm2(21)(22)(23)(24).

The volume of PRP to be used may vary from a minimum of 0.5 ml to a maximum of 2 ml according to published data in literature (25).

Patients who met the inclusion criteria undergone laparoscopic surgery, where one trocar of 5 mm was introduced at the level umbilical for the optic and other two of 5 mm were positioned in the right and left iliac fossa. The surgery was performed, in general anesthesia, by a skilled gynecologist surgeon. The average duration of the surgery was 40 ± 10 minutes. The CO2 laser was inserted through one of the secondary trocars, it was used to create microdissections at the ovarian subcortical, with an emission of waves with a wavelength of 10.6 μ, at a distance of 5-10 mm and at a depth of 4-10 mm. The final number of dissections cannot be calculated because it depends on the size of the single treated ovary.

The injection of PRP was performed into the subcortical space of the ovaries, using a needle, with the advantage of proceeding to multiple infiltrations under direct laparoscopic vision.

Special attention was paid to all possible complications and side effects of the two procedures.

After the surgical phase, patients undergone a double ovarian stimulation (DuoStim protocol): the first one was performed during the follicular phase of the first menstrual cycle after the laparoscopy and the second one during the luteal phase of the same cycle.

The ovarian stimulation involved the administration of highly purified menotropin (human menopausal gonadotropin, HMG) for a dosage of 300 IU/d hmg (Meropur, Ferring Pharmaceuticals, Germany), until the follicular maturation criteria are met (at least 3 follicles with a diameter of 17 mm). A oocyte pick-up for each stimulation was planned.

Daily administration of GnRH antagonist Ganirelix (Orgalutran; Msd Italy Srl), with flexible protocol, at a dose of 0.250 mg/day was started in the presence of at least 1 follicle with a medium diameter range of 14 mm. A single injection of 2 vials of GnRH agonist (Decapeptyl, Ipsen, Signes, France) 0,1mg/ml was administrated at 36 hours before to proceed to the first egg pick-up (OPU-1). After 5 days from the first oocytic sampling (OPU-1) a second stimulation cycle was started with the same drug protocol. The second egg sampling (OPU-2) was carried out after an average of 15 days from the OPU-1.

Concerning the CO2 Laser, it is a device able to determine a specific biological effect on tissues. The CO2 laser is known to be used in treatment and prevention of vulvo-vaginal atrophy in patients in menopause (11)(12). These results are achieved thanks to the transfer, in the vaginal walls, of thermal energy that determines a series of effects at the tissue level including: the proliferation of the epithelium rich in glycogen, neovascularization, the synthesis of collagen and components of the cellular matrix (13)(14).

Based on this application experience, it is reasonable to translate these results on other tissues that can undergo a “progressive process of atrophy and exhaustion” of the their physiological function. Laser action on ovarian tissue could induce a neovascularization and reactivation of cellular components in the perifollicular microenvironment.

Laser CO2 method has been used, 30 years ago, in the treatment of ovarian polycystosis, according to technique drilling, to restore a physiological function ovarian endocrine, with any complications (15)(16)(17).

It was an effective and safe treatment in subfertile women with syndrome of the polycystic ovary resistant to clomiphene, reducing the number of multiple pregnancies and the incidence of OHSS.

Until now there are no published studies concerning the effects of the combination of the CO2 laser and PRP and therefore this study would be the first to use this type of innovative treatment.

# MATERIALS AND METHODS

The study is an open Single Arm Interventional Clinical Study, where the number of sample size (n. of patients: 30) was evaluated with the relative power test and the adequacy of the statistical sample, in order to establish the feasibility and safety of the procedures for each patients, and also to obtain information to determine the sample size for a subsequent larger-scale study.

The study had a "Intention To Treat" approach for the analysis of collected data, in order to monitor also possible drop out cases from the study.

It was conducted in the Department of the Pathophysiology of Human Reproduction (IVF Center) in the Hospital Santa Margherita La Fratta, Cortona (Tuscany, Italy), from January 2021 to January 2022.

We enrolled patients of childbearing age, between 18 and 43 years, with poor ovarian reserve diagnosed with AFC <5-7 follicles and /or altered hormonal levels (FSH> 12 mIU / ml; AMH <0.5 ng / ml) and / or with at least one IVF cycle that has given failed oocytes recovery (18)(19)(20).

Once obtained the informed consent, patients have been subjected to a hormonal sampling performed on the 2nd day of the menstrual cycle for the evaluation of FSH and AMH and to an ultrasound examination performed with the aim to evaluate the antral follicles (antral follicle count or AFC) in both ovaries. The AFC was expressed in numerical value.

The efforts to overcome a poor response have so far been focused on stimulating the ovaries to promote follicle growth. Many controlled ovarian stimulation (COS) protocols and other alternatives have been tested, but none have proven to be successful, probably due to the low number of follicles inside the ovary or due to reduced activity or reduced sensitivity of the local receptors. However, a small reserve of quiescent primordial follicles remains, even in ovaries of menopausal and POF patients, which could potentially contribute to yield final oocytes. In fact, oocytes could be recovered after the activation and growth of these "dormant" follicles (26).

PRP has been studied as long-used product in the field of infertility. Kim *ET AL*. have investigated the potential effects of intra-ovarian PRP administration on the POR patients and they concluded that total oocytes count showed a statistically signifcant increase in all POSEIDON groups with more notable changes in group 4 Interestingly, this study showed that 9.75% of women got pregnant following a single session of PRP injection without the need of a subsequent IVF cycle. However, it was less than 14.75% for those cases undergone assisted reproductive medicine (27).

Hajipour *ET AL*. have mentioned in their systematic review that growth factors in the PRP could afect, in a positive way, diferent characteristic features of oocytes to eventually increase survival rate of follicles compared to the controls (9).

Also Cakiroglu *ET AL*. investigated the results of intra-ovarian PRP administration on primary ovarian insufciency, concluding that PRP treatment could lead to increased antral follicle count, improving ovarian function after the PRP treatment (28).

No significant change was observed in AMH value and this matter justifies that the outcomes of patients are more likely achieved through a non-hormonal pathway(s).

From the analysis of the published studies concerning the application of PRP, a phase I pilot project is about 8 women in perimenopausal status, with an average age of 45.1 ± 4.4 years, treated with autologous PRP, administered in the ovary through a transvaginal injection guided by ultrasound. The ovarian rejuvenation was successfully achieved in the 8 cases treated, confirmed by the restoration of the menstrual cycle 1 - 3 months after PRP treatment. In addition, in all the treated cases, 2.5 ± 0.71 follicles with a diameter of 15.2 ± 2.05 mm have been successfully recovered and 1.5 ± 0.71 oocytes and 1.50 ± 0.71 MII oocytes were retrieved. These have been inseminated through the ICSI technique and the resulting embryos have been cryopreserved at stage 2pn (22).

Scott *ET AL*. published a phase I clinical trial conducted on 4 women in menopause and perimenopause, treated with about 5 ml of autologous PRP, activated with calcium gluconate, administered in the ovary through a guided transvaginal ultrasound injection. AMH, FSH and estradiol values were monitored at 2-week intervals after PRP treatment and compared to baseline levels. A statistically significant reduction of FSH ($p < 0,01$) and 5.3 ± 1.3 MII oocytes were collected. The IVF technique was carried out at a distance of 78 ± 22 days from PRP treatment. Each patient has obtained at least one blastocyst, available for cryopreservation. In addition, none of the treated women developed complications as a result of experimental treatment (23).

Sills *ET AL* conducted a prospective clinical trial where 182 patients with low ovarian reserve and/or at least 1 failed in vitro fertilization (IVF) cycle were treated with autologous PRP (about 5 ml) activated with calcium gluconate, administered in the ovary through a guided transvaginal ultrasound injection. The control group is represented by patients treated with conventional hormonal stimulation, having homogeneous characteristics to those of the experimental cohort group. Authors observed improved serum AMH in 51 patients (28%) with median increase of 167% [95%CI 91; 280] after treatment; the mean interval to maximum AMH increase was 4 weeks (range 2-10 weeks). Improved post-treatment AMH was not limited to younger patients; when stratified by age (<42 vs. ≥42yrs), significant AMH improvements were seen in both groups after treatment ($p=0.03$ and 0.009, respectively) (29).

To date the application experience of EunT does not guarantee the accurate deposition of the preparations at the level of the virtual cellular spaces of the ovarian subcortical, but it highlights an improvement in the ovarian function of the treated patients, without manifesting any adverse effect (acute and/or chronic toxic effects) (10)(21).

All the studies concerning the application of PRP and the improvement of ovarian reverse used a standard procedure for the intraovarian injection of PRP, that is by the transvaginal approach. No data are available on the application thruogh laparoscopy and no sperimental data are on the application of $CO_2$ laser to improve the effectiveness of the action of PRP on the ovarian function.

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 long-standing 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.

The thermal energy can determine a series of effects at the tissue level without damage.The rational on the use of biotherapy lies in the laser action on ovarian tissue with induction of a neovascularization and consequent reactivation of the factors present in the microenvironment intercellular that lead to the reactivation of the dormant follicles by the stabilization of the cellular microenvironment, the neoangiogenesis and the activation of gametes cell receptors, stimulating hormonal feedback to ovarian tissue.

# RESULTS

The characteristics of the population were an average age of 39 ± 2.5 years, a body mass index

(BMI) of 23 ± 2.9, a time of infertlity of 3.7 ± 3 years, a basal AMH of 0,5 ± 0,22 ng/dl, a basal FSH of 17±12.2 and a AFC of 2.5 ± 1,2.

The sperimental applicaion of the two techiques showed to be a safe procedure for all enrolled patients.

No important complication or side effect were registred. Only one patient had a fever 48 hours after surgery, a self-limited episode in a few days, with no hospitalization. The average duration of the surgery was 40 ± 10 minutes. It lasted longer in the 30% of cases (9 of 30 patients), where previous abdominal surgery like ovarian cystectomy or appendicectomy was perfomed, thus more time was needed for the lysis of adhesions. No iatrogenic bleeding was observed after the CO2 laser microdissection performed on the ovaries. Induction of follicular growth is a milestone in the recovery of ovarian functional activity in which the quiescent follicles are re-activated and sensitized to exogenous induction with gonadotropin. According to the results of this study and according to the fact that CO2 laser and intraovarian injection PRP separately proved irrefutably a remarkable regenerative capacity on various types of human tissue, it is resonable that the combination of the two technique can induce improvement of the ovarian reserve in term of laboratory test (an increase of AMH and a reduction of FSH) and the quantitative improvement of antral follicular count.

Follow up period of our enrolled patients is ongoing, they are undergoing office visits and transvaginal ultrasound and blood sample for hormonal evaluation, in order to monitor the patients health status and the final data of effectiveness of the treatment in terms of cumulative pregnancy rate and live birth rate.

# CONCLUSION

Our study showed that the combination of the two techniques is safe, with no risk for patients. The rational of the propose was that both the CO2 laser and the application of PRP are correlate to beneficial effects. Another important matter is that the injection of PRP in the subcortical ovarian space can be more precise, under laparoscopic vision rather than by a transvaginal guidance.

Data relative to the efficacy of the treatment on the improvement of ovarian reserve parameters and the subsequent artificial reproductive technique (ART) cycle outcomes will be pubblicate in a future manuscript. However, according to published data, it is well known its positive relation, particularly in women with poor ovarian response. The improvement of ovarian function was registred in term of total oocyte number and especially metaphase (MII) oocytes. PRP injections are effective and safe to improve markers of low ovarian reserve prior to ART cycle. On the other hand, ormonal level seems to change not signifcantly. This might be a clue to how PRP did not exploited the hormonal pathways but maiby there are involved other supposed mechanisms such as the angiogenesis.

PRP seems to be the appropriate treatment for the patients with poor ovarian response who show resistance to the hormonal treatments but further evidence is required to evaluate the impact of combination of Co2 laser and PRP as a potential better treatment to obtain pregnancy.

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