Official Title: Follicle Activation in Patients with Poor Ovarian Response Through Fragmentation of the Ovarian Tissue.

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Study Protocol

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1. Scientific background

Assisted reproductive techniques (ART) are increasingly used to treat infertility. In vitro fertilization (IVF) involves pharmacological stimulation of the ovary in a supra-physiological manner with gonadotrophins to obtain multiple follicles. However, many women do not respond adequately to the usual treatment protocols during controlled ovarian hyperstimulation. These patients have been classified as "low responders" and have lower gestation rates compared to "normoresponders", ranging from 0 to 18% (Tarlatzis et al., 2003). The incidence of low ovarian response in IVF treatments is estimated to be around 10-25% (Pellicer et al., 1998). The wide range of prevalence described in the literature is explained by the lack of consensus on the specific clinical criteria that have been used to define low response. Given the disparity of criteria, the ESHRE (European Society of Human Reproduction and Embryology) established a consensus definition for low response:

- Option 1: two episodes of poor ovarian response (≤ 3 oocytes retrieved on a conventional schedule) in the absence of advanced maternal age or evidence of low ovarian reserve.
- Option 2 at least two of the following: age ≥ 40 years or any other risk factor for poor ovarian response, previous IVF cycle with ≤ 3 oocytes (following a conventional stimulation protocol), abnormal ovarian reserve test (antral follicle count of ≤ 5, AMH < 0.5 - 1.1 ng/mL).

Pathophysiological basis of low response

- Ovarian reserve: the concept of low responder is closely related to that of ovarian reserve. Ovarian reserve is defined as the number and degree of quality of follicles remaining in the ovary at a given time (Broeckmans et al., 2006) and is the main determinant of ovarian response to gonadotrophin stimulation (Pellicer et al., 1998).

  - Other causes: a group of young women with low ovarian response have normal basal FSH hormone levels in whom there is no apparent cause for this response (Tarlatzis et al., 2003). Many hypotheses have been proposed to explain this condition. However, none have been proven: a decrease in the number of FSH receptors available in the granulosa cells, FSH post-receptor defects, etc.

Strategies aimed at improving the management of responding casualties

Efforts to provide better control of responding casualties have currently focused on a priori identification of responding casualties and modification of existing protocols themselves.

A priori identification of responder casualties

- Antral follicle count by transvaginal ultrasound: Antral follicle count is nowadays considered the best predictor of ovarian response in isolation (Broeckmans et al., 2006). Most authors use as a cut-off point 3-4 antral follicles on the day when pituitary suppression is proven.
- FSH elevation at the beginning of the cycle: In disuse because of its variable sensitivity and specificity.
- Maternal age: The probability of not conceiving the first child within one year increases from 5% in women in the 20-30 age group to 30% in women over 35 (Broeckmans et al., 2006).
- Anti-Müllerian hormone (AMH): recently, it has been proposed that the determination of AMH) in blood could be used as a test of ovarian reserve so that decreased levels would indicate ovarian aging. Its efficacy is similar to that of the antral follicle count (Broeckmans et al., 2006).

Therapeutic modifications aimed at reducing the incidence of low response

Since the beginning of ART application, many different protocols have been proposed to manage patients with low response, obtaining very different results in the further studies published, making it very difficult to conclude them. Increasing the dose of FSH used (Tarlaltzis et al., 2003), the use of recombinant FSH instead of purified urinary FSH (Raga et al., 1999), the use of FSH in the luteal phase (Rombauts et al., 1998), modifications in pituitary desensitization protocols (García-Velasco et al. 2000), or pre-treatment with estrogens or combined oral contraceptives (Fanchin et al., 2005), pre-treatment with androgens (Fábregues et al.,2009). The reality is that none of these interventions have been shown to be effective in increasing the gestation rate in the subgroup of low responders.

New strategy for ovulation stimulation

In every woman, even in the low responder, there is a pool of dormant (primordial) follicles, not stimulable with gonadotrophins, which, if they could be used would significantly increase the functional reserve of the patient. Aaron Hsueh's research group described a follicular activation technique (Kawamura et al., 2013), which consists of activating these primordial follicles and converting them into antral (stimulable) follicles before the IVF cycle is performed by fragmenting the ovarian tissue. Fragmentation of the ovarian cortex increases actin polymerization and disrupts the intracellular Salvador/Warts/Hippo (SWH) signaling pathway. The SWH pathway is responsible for maintaining proper organ size. Its disruption leads to increased cell growth and survival because CCN (growth factors) and BIRC (inhibitors of apoptosis) levels are increased, resulting in activation of primordial follicles. This explains why the final consequence of ovarian fragmentation is the growth of ovarian primordial follicles and

therefore the generation of a greater number of antral follicles, which are susceptible to stimulation with gonadotrophins, thus potentially increasing the number of mature oocytes that could be obtained in patients undergoing the technique.

From a practical point of view, by laparoscopic surgery, the ovarian cortex is removed, mechanically stimulated in vitro (the cortex is fragmented into multiple aliquots), and then reimplanted, and the conventional IVF cycle is carried out.

This technique was tested in 27 patients with primary ovarian failure (early menopause, the paradigm of ovarian unresponsiveness). The results were as follows: eight patients presented follicular growth, obtaining mature oocytes from five of them and achieving pregnancy in one patient who finally gave birth to a healthy baby. It should be noted that this study was carried out in a "worst-case scenario", in menopausal patients of whom only thirteen had residual follicles in the histological analysis of the pieces, and the eight in whom ovulation was induced belonged to this group, which represents a 62% response to treatment.

2. Hypothesis

The fragmentation of the ovarian cortex in vitro activates the growth of primordial follicles, leading to the appearance of a greater number of antral follicles, which would make it possible to obtain more oocytes after controlled ovarian stimulation in patients with low ovarian response, which would be useful for improving the success rate of IVF in patients with low ovarian response.

3. Objectives

Main objective:

- To assess whether the fragmentation of ovarian cortex increases the pool of antral (potentially stimulable) follicles.

Secondary objectives:

- To analyse whether the proposed intervention improves ovarian response after gonadotrophin stimulation.
- To evaluate the pregnancy rate.

4. Study design

TYPE OF STUDY

Two-phase study:

- Phase 1: Randomised, controlled, single-blinded clinical trial. Sixty patients previously confirmed as low responders according to ESHRE criteria will be randomised to two treatment arms after assessment of ovarian reserve:
    - Arm 1 (control): no intervention was performed on the patient.
    - Arm 2 (intervention): removal of ovarian cortex by unilateral laparoscopic oophorectomy, fragmentation of ovarian tissue into 2 mm^2 aliquots. The ovarian tissue was then re-transplanted under the serosa of the tube homolateral to where the cortex was removed.

  Subsequently, bi-weekly ultrasound monitoring was performed to determine the number of antral follicles present in both the native ovary and the re-implanted ovary to determine the number of antral follicles. The number of antral follicles in both intervention groups was compared and the number of antral follicles in the native ovary was also compared with the reimplanted one in each of the patients.

- Phase 2 Prospective observational study. A cohort of all patients who participated in phase 1 was formed and followed for six months. Those patients who wished to undergo IVF treatment received standard treatment for low responders. The final objective consisted of assessing the gestation rate in both groups, as well as the parameters related to the reproductive technique.

STUDY POPULATION

Patients affected by infertility requiring treatment by IVF or ICSI and who were confirmed low responders based on the inclusion criteria.

INCLUSION CRITERIA (one must be met)

- At least two episodes of poor ovarian response (≤ 3 oocytes retrieved in a conventional scheme).
- A previous IVF cycle with ≤ 3 oocytes (following a conventional stimulation protocol) and presence of an abnormal ovarian reserve test (antral follicle count of ≤ 5 or AMH ≤5pM).

EXCLUSION CRITERIA (the presence of one excludes from participation)

- Patients older than 40 years.
- Clinical suspicion of endometriosis.

- Anovulatory patient (defined by the presence of irregular cycles and serum progesterone on day 21 of the cycle of ≤ 10 ng/mL).
- Couples with severe male factor: severe oligoasthenozoospermia, oligoasthenoteratozoospermia and azoospermia.
- All patients who do not voluntarily give their express written consent.

STUDY VARIABLES

PHASE 1

- Antral follicle count at randomisation (per ovary).
- Antral follicle count after intervention/control (per ovary, fortnightly for 6 months).
- Determination of AMH and FSH/E_2 at the time of randomisation.
- AMH, FSH/E_2 determination after intervention/control (per ovary, monthly for 6 months).

PHASE 2

- Number of metaphase II oocytes obtained after ovarian stimulation.
- Number of follicles punctured.
- Total number of oocytes obtained after ovarian stimulation.
- Number of fertilised oocytes.
- Evolution of embryo development.
- Serum oestradiol levels on the day of hCG.
- Total dose of FSH used.
- Necessary days of gonadotrophin stimulation.
- Clinical pregnancies (determined as the presence of the sac after embryo transfer).
- Progressive gestations (those exceeding 12 weeks).

5. Methods

ASSESSMENT OF OVARIAN RESERVE

- Basal and follicular serum determination of steroid hormones: serum determinations of FSH, E_2 and AMH) were performed with a microparticle immunoassay kit integrated into the ABBOTT AxSYM® Plus system and 5.20 software (Abbott Laboratories, Abbot Park, IL, USA).

- Ultrasound determinations: antral follicle counting was performed with a GE iVoluson (General Electric, Spain) equipped with 3D vaginal transducer (6.5-9MHz). Antral follicles were counted on day 2-5 of the menstrual cycle.

REMOVAL AND REIMPLANTATION OF THE CORTEX

The removal of the cortex was performed by tri-port laparoscopic surgery. Under general anaesthesia, unilateral oophorectomy was performed by cutting and bipolar coagulation of the infundibulum-pelvic and utero-ovarian pedicles. For reimplantation of the cortex after fragmentation, the same laparoscopic approach was used as for removal, in order to avoid unnecessary incisions. The incubated tissue was placed in a peritoneal window in the serosa of the tube homolateral to the removed ovary.

FRAGMENTATION AND INCUBATION OF THE CORTEX

After removal of the ovary, the ovary was decorticated (separation of the medulla) by friction with a cold scalpel. The resulting cortex was fragmented into small portions of approximately 1-2 mm^2.

CONTROLLED OVARIAN STIMULATION

Those patients who undergo assisted reproduction treatment during the phase 2 follow-up process included stimulation with high doses of gonadotropins (Antagonist+350 FSH+150 LH).

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