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ASSISTED REPRODUCTION TECHNOLOGIES

Accumulation of oocytes from a few modified natural cycles to improve IVF results: a pilot study

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Abstract
Purpose To evaluate the role of co-transfer of embryos derived from vitrified oocytes accumulated during the previous modified natural cycles and an embryo developed from the last one as an alternative to repetitive single embryo transfer in a fresh modified natural cycle.
Methods Thirty-six patients underwent ICSI procedure with three frozen natural oocytes supplemented by a fresh one obtained from the fourth modified natural cycle. Thirty-one controls received at least three consecutive single embryo transfer in a fresh modified natural cycle.
Results In the study group the oocyte retrieval, survival and total fertilization rate were 73.0 %, 78.1 %, and 64.5 %, respectively. Fifty-two embryos were transferred in 29 transfers. In the control group the oocyte retrieval and fertilization rate was 77.4 % and 83.7 %, respectively. Fifty single embryo transfers were performed. Of a total 14 pregnancies obtained in the study group 10 were defined as clinical and 4 as abortions. In the control group a total of 8 single clinical pregnancies and 2 miscarriages were encountered. The overall (20.0 % vs 48.2 %) and the clinical (16.0 % vs 34.4 %) pregnancy rate were significantly higher in the study group having cumulative embryo transfer following the oocyte accumulation.
Conclusions These data demonstrate that the co-transfer of embryos derived from vitrified oocytes accumulated during the previous modified natural cycles and an embryo developed from the last fresh modified natural cycle assure an excellent clinical outcome with the overall and clinical pregnancy rate significantly higher compared to the repetitive single embryo transfer in a fresh modified natural cycle.

Keywords Oocyte vitrification · Natural cycle · Clinical efficacy · ICSI

Capsule The co-transfer of embryos derived from vitrified natural oocytes and an embryo developed from the fresh natural cycle assure an excellent overall and clinical pregnancy rate.

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Introduction

The first successful IVF procedure was performed in an unstimulated menstrual cycle [1]. However, with the use of exogeneous gonadotropins and GnRH-agonists IVF in natural cycle has been largely replaced by IVF with ovarian stimulation. The aim of controlled ovarian hyperstimulation is to reduce the cycle cancellation rate, increment the number of oocytes and top-quality embryos and ensure better results in terms of pregnancy rates [2–5]. However, in different clinical situations such as reduced ovarian reserve, previous oncologic or estrogen-dependent disease the natural cycle continue to be considered the method of choice for IVF treatment [6–16].

This type of IVF protocol appears to be safe due to exogenous gonadotropins avoidance and patient-friendly because of minimal medication, easy oocyte retrieval, no risk of OHSS and possible repetition in consecutive cycles [2, 3, 5]. In addition, the natural cycle IVF minimizes physical and emotional stress for the patients [17] and reduce the cycle costs about 20–23 % compared to stimulated protocols [18, 19]. Moreover, a mean birth weight of newborns from natural cycle has been found to be a significantly higher than in controls probably reflecting the better neonatal health [20].

However, the pregnancy rate per started cycle have been observed to be relatively low (0–18.3 %) [2, 3, 14, 21–24]. The cumulative pregnancy rate in the population of normal responders was estimated about 20.8 % [3] and 44 % [4] only after three and nine cycles, respectively. Life-table analysis in

three studies showed cumulative pregnancy between 41.7 % and 46.0 % [18, 19, 25] after 3–5 started cycles.

The study published by Cobo et al. [26] demonstrated that accumulation of vitrified metaphase-II oocytes obtained from multiple stimulation cycles and their insemination all at the same time lead to the high life-birth rate comparable to that observed in women adequately responding to ovarian stimulation. However, the strategy of repetitive stimulations is not applicable in all patients.

Therefore, the accumulation of oocytes obtained from repeated modified natural cycles (MNC) supplemented by the fresh one from the last cycle and their simultaneous insemination in order to obtain the number of embryos comparable to those transferred after ovarian stimulation may represent a potential alternative for women with contraindications or who simply wish to avoid the hormonal treatment. In this study we report our preliminary experience with this novel protocol.

Material and methods

The study involved 67 women undergoing IVF procedure between May 2010 and July 2012. Thirty-six patients followed the study protocol in which the ICSI procedure was based on three frozen natural oocytes supplemented by a fresh one, if available, obtained from the fourth MNC. The control group was represented by 31 patients who underwent at least three consecutive MNC followed by a simultaneous single embryo transfer.

Inclusion criteria were the presence of any contraindication to the control ovarian stimulation, reduced ovarian reserve or women preference for more patients' friendly protocol avoiding the multiple follicular growth. The reduced ovarian reserve was defined as abnormal ovarian test results (antral follicle count <7 and/or AMH <1.1 ng/ml) and recovery of less than 3 oocytes in previous conventional stimulation. The only exclusion criteria were a history of ovulation disorders. An informed consent was obtained from all participants. The patients' and controls' characteristics are presented in Table 1.

The spontaneous follicular growth in the MNC was monitored by ultrasound starting on cycle day 3 to exclude the presence of cystic structures. The frequency of subsequent controls was continuously adapted according to the follicular increment. In order to avoid premature ovulation GnRH-antagonist ganirelix (Orgalutran, Organon, Rome, Italy) was started at daily dose of 0.25 mg when follicle reached the mean diameter of 14 mm and was continued until the ovulation induction planed at follicular mean diameter of 17 mm. The ovulation was induced with 10,000 IU of HCG (Gonasi, Amsa, Rome, Italy) and oocyte retrieval was performed 36 h after HCG administration under transvaginal ultrasound-guided puncture of the follicle.

The oocytes were vitrificated by three-step gradient cryoprotectant loading process using the Kitazato Safety Vitrification Kit (Cryotop and Vitrification Kit, Kitazato BioPharma Co., Japan). The oocytes were placed in a 40 μl drop of basic

Table 1 Baseline characteristic of the study population

	Study group	Control group	*P* value
Female age (yr)	38.2±3.46	39.9±3	ns
Female age distribution			
≤34 years	1	0	ns
35–40 years	18	15	ns
40–45 years	17	16	ns
AMH (pmol/l)	6.3±7.7	5.8±4.9	ns
BMI (kg/m^2)	21.8±4.3	22.2±1.8	ns
Antral follicle count (n°)	5.0±3.2	4.0±2.9	ns
Type of infertility (%)			
Male	3/36 (8.4 %)	3/31 (9.7 %)	ns
Female	10/36 (27.7 %)	8/31 (25.8 %)	ns
Male and female	23/36 (63.9 %)	20/31 (64.5 %)	ns
Cause of female infertility (%)			
Reduced ovarian reserve	20/36 (55.6 %)	19/31 (61.3 %)	ns
Oncologic disease	3/36 (8.4 %)	0/31 (0 %)	ns
Endometriosis	3/36 (8.4 %)	2/31 (6.4 %)	ns
Uterine fibroids	1/36 (2.8 %)	2/31 (6.4 %)	ns
Coagulative disorders	1/36 (2.8 %)	0/31 (0 %)	ns
Multiple causes	5/36 (13.9 %)	5/31 (16.1 %)	ns

solution for 2 min, then the drop was bridged into the equilibration solutions for 2 min and replaced to a new 40 μl drop of equilibration solutions for another 2 min. Then the oocytes were equilibrated for 10 min in a 0.3 ml of equilibration solution and finally placed in 0.3 ml of vitrification solution for 1 min. In the next step the oocytes were loaded on a Kitazato Cryotop and immediately plunged into liquid nitrogen for storage. All the procedure was performed at room temperature with the media being prepared 30 min in advance.

The warming procedure (Cryotop and Vitrification Kit, Kitazato BioPharma Co., Japan) started with the cryotop removal from liquid nitrogen and its immersion in 1 ml of thawing solution for 1 min. The oocyte was then transferred into 0.3 ml of dilution solution for 3 min and equilibrated in 0.3 ml of washing solution for another 10 min. Finally the oocytes were incubated in culture media for 2 h at 37 °C, 6.0 % CO, 5 % O2 before ICSI. The first step of the thawing procedure was performed at 37 °C, while the others two were effectuated at room temperature [27].

Insemination was performed by ICSI as previously described [28]. Fertilization was assessed 16–18 h after ICSI. On this occasions zygote morphology was evaluated according to criteria based on the assessment of the number and distribution of nucleolar precursor bodies in the pronuclei. The two-pronucleate zygotes were cultured under the same conditions for two additional days. At the time of medium change on day 2 and 3, the embryos were assessed again with the previously described scoring criteria [28]. Sperm-injected oocytes, zygotes and embryos were incubated at 37 °C in G-FERT (Vitrolife, Goteborg, Sweden) equilibrated with 6 % CO2 in air. Embryos were transferred 3 days after ICSI with the use of K-JETS-7019-SIVF embryo-transfer set (Cook, Queensland, Australia).

The luteal phase was supported with natural micronized progesterone (Prontogest, Amsa, Rome, Italy) administered by intramuscular route in one daily dose of 50 mg. The treatment was started on the day of oocyte collection and continued until the day of the pregnancy test. In case of a positive test, the therapeutic regimen was continued during the first pregnancy trimester. Clinical pregnancy was defined as the presence of a gestational sac with positive heartbeat.

A statistical analysis was performed using an SPSS (SPSS, Inc., Chicago). The comparison of qualitative data were carried out with Fisher test. The significance level was defined as 0.05 and a value of $p<0.05$ was considered statistically significant.

Results

The demographic and clinical characteristics of the study population and controls are presented in Table 1. Basic patients characteristics including age and different causes of infertility distribution were comparable in both groups. The ICSI procedure was performed with frozen-thawed spermatozoa obtained from testicular sperm extraction only in one case of each study group.

The 178 egg retrievals performed in 36 women undergoing the accumulation program leaded to the collection of 130 oocytes of which 18 could not be used for IVF procedure because of poor morphology or immaturity whereas 87 have been cryopreserved. The 25 eggs obtained from the last MNC have been used as fresh one at the time of cumulative ICSI. The retrieval rate of oocytes was 130/178 (73.0 %). The survival rate of frozen-thawed oocytes was 78.1 %. A total of 93 (25 fresh and 68 frozen) oocytes were injected and 60 (25 fresh and 35 frozen) fertilized (64.5 %). The fertilization rate for fresh and vitirified oocytes were 100.0 % and 53.8 %, respectively. Fifty-two embryos were transferred and 4 cryopreserved as supernumerary ones (Table 2). In this group 3 patients transferred embryos from only fresh oocytes, 9 from only frozen oocytes and 17 from both type of eggs.

The 102 egg retrievals performed in 31 controls leaded to the collection of 79 oocytes of which 74 were injected and 62 fertilized. The oocyte retrieval and fertilization rate was

Table 2 Biological outcomes study group and control

	Study group		Control	
	Total N°	Mean ± SD	Total N°	Mean ± SD
Frozen oocytes	87	2.4±2.3	–	–
Survived oocytes	68	1.88±1.2	–	–
Fresh oocytes	25	0.69±0.46	79	0.82±0.4
Injected oocytes	93	2.62±1.3	74	0.9±0.7
Fertilised oocytes	60	1.7±1.4	62	0.8±0.3
Trasferred embryos	52	1.4±1.2	50	0.7±0.5
Excellent quality embryos	35	1.0±0.5	31	0.5±0.5
Good quality embryos	11	0.21±0.64	11	0.2±0.5
Poor quality embryos	6	0.17±0.45	8	0.1±0.3

77.4 % (79/102) and 83.7 % (62/74), respectively. Fifty single embryo transfers were performed (Table 2).

Of a total 14 pregnancies obtained in the study group 10 were defined as clinical (6 single and 4 twin) and 4 as abortions. In the control group a total of 8 single clinical pregnancies and 2 miscarriages were encountered. The clinical outcome of our study population and controls are showed in Table 3. The overall (20.0 % vs 48.2 %) and the clinical (16.0 % vs 34.4 %) pregnancy rate were significantly higher in the study group having cumulative embryo transfer following the oocyte accumulation (Table 3). In addition, in 9 patients transferring 12 embryos from only vitrified oocytes 3 clinical pregnancies and 1 miscarriage were observed (pregnancy and implantation rate of 44.4 % and 33.3 %, respectively).

No complication related to the oocyte retrievals or embryo transfers were encountered. All clinical pregnancies resulted in the delivery of healthy babies. In addition, 9 patients underwent amniocentesis and all foetuses were verified to have normal karyotypes.

Discussion

The results of this study showed that transfer of embryos derived from vitrified natural oocytes supplemented with a fresh one from the last MNC leads to an excellent clinical outcome with overall pregnancy and implantation rate of 48.2 % and 32.7 %, respectively. These results appears to be comparable to cumulative pregnancy rate reported by others authors after six to nine single embryo transfers in spontaneous cycle [3, 4] or after transfers of fresh and frozen embryos in stimulated programs [29]. Moreover, unique multiple embryo transfer reduce the entity of patients' stress related to the pregnancy attendance compare to consecutively repeated single embryo transfers. In addition, the avoidance of drug and reduced abstinence from normal work activity after each single transfer reduce the social and medical costs of treatment.

Some reports claim a lack of benefit with higher oocytes number probably due to the detrimental effect of elevated steroid levels on oocytes quality, embryos aneuploidy and endometrial receptivity [5, 30, 31]. The biological advantages of natural cycle include the presence of a single oocyte of potentially better quality allowing the transfer of a healthier embryo into a more receptive endometrial environment [32]. Thus, the high clinical success of this study may be explained by a more receptive uterine environment, higher trophoblast and/or embryos genetic quality deriving from spontaneous follicular growth in absence of ovarian stimulation.

It has been suggested that, in relation to the cryopreservation method adopted, the loss of the overall reproductive potential of frozen oocytes occurs as a consequence of poor survival, fertilization and cleavage rates leading to a high cancellation of cycles [33]. Thus, until recently, cryopreservation of human oocytes was defined as generally inefficient procedure and its application was restricted to the very special situations [29, 34–37]. The slow freezing remains widely used for oocyte storage but vitrification has progressively emerged as an alternative technique [29, 33, 37–41]. This procedure limits the problem of cryoinjury by avoiding ice crystal formation and exerts a less detrimental effect on meiotic spindle [29, 33, 39, 40, 42]. In fact, Tulandi et al. [35] have suggested that the pregnancy rates after oocyte vitrification are approximately twice as high as after traditional freezing (10–21 % vs 21–45 %, respectively). In particular, Kim et al. [33] observed 285 vitrified oocytes documenting a 72 % of fertilization and 45 % of implantation rate.

Morover, the preliminary estimation of the incidence of birth anomalies found in babies born after vitrified oocytes does not appear different from that of infants born through natural conception. The Review of all cases of oocytes vitrifications from 1986 to 2008 reports 392 live born babies and only 6 anomalies (1.5 %) [41]. Based on observation of 221 children born after oocyte vitrification the mean birthweight reported was 2,920 g for singletons and 2,231 g for multiples. The low birthweight rate among singletons was 18 % and among multiple 80 %. The premature delivery rate was 26 % for singletons and 71 % for multiple pregnancies [43]. However, long-term child follow-up studies are lacking.

These findings reported by the literature influenced our choice of cryopreservation method. In this study the survival and fertilization rate of vitrified oocytes was 78.1 % (68/87) and 51 % (35/68), respectively. The fertilization rate of fresh oocytes was higher. However, women receiving transfer obtained from only vitrified oocytes had still a good pregnancy (44.4 %) and implantation rate (33.3 %). In addition, 4 of 14 (28.5 %) of pregnancies were twin confirming an unmodified reproductive potential of vitrified oocytes.

Many factors are thought to affect IVF success rate such as women age, serum FSH level, number of injected oocytes, semen quality and number of top-quality embryos [44–47]. The controlled ovarian stimulation followed by recovery of high number of oocytes obtained from multiple follicles allows to optimize the IVF cycle. However, a question has been

Table 3 Clinical outcomes of study population and control

	ET from fresh and frozen oocytes ($n=29$)	ET from only fresh oocytes ($n=50$)	P value
All pregnacies	14/29 (48.2 %)	10/50 (20.0 %)	$P<0.01$
Clinical pregnancies	10/29 (34.4 %)	8/50 (16.0 %)	$P<0.03$
Miscarriages	4/29 (13.7 %)	2/50 (4.0 %)	ns
Implantation Rate	17/52 (32.7 %)	10/50 (20.0 %)	ns

raised about a reasonable number of oocytes needed for the pregnancy achievement [48].

A recent meta-analysis suggested that the number of oocytes needed to achieve a satisfactory implantation rate depends on the ovarian stimulation protocol used. Five retrieved oocytes were associated with an ideal ongoing pregnancy rate (67 %) per embryo transfer in patients undergoing a mild stimulation protocol using GnRH-antagonist [49, 50]. On the other hand, a development of less than five follicles in a standard stimulation protocol is usually associated with poor outcome related to the low number transferred embryos and high cancellation rate due to the fertilization failure [51].

Because of the specific nature of MNC this type of ovarian preparation for IVF may be partially comparable only to the mild ovarian stimulation and the decision of four oocytes to be used was based on the literature date regarding the optimal number of eggs in the program of mild-ovarian stimulation protocol. The literature regarding application of repetitive natural cycle IVF reports an elevated drop-out rate up to 47.8 % [4]. Therefore, the limitation of oocyte number to four was undertaken with the aim to guarantee the patient-friendly treatment and prevent the drop-out.

The Cochrane Database Systematic Review [52, 53] suggested that elective single embryo transfer is associated with a lower clinical pregnancy and live birth rate than double embryo transfer. The review by Polyzos et al. [14] suggested that pregnancy outcome in poor responder women undergoing the natural IVF were substantially low with a positive HCG rate of 4.6 % and live birth of 2.6 % per cycle. On the other hand, the study of Jonsdottir et al. [54] confirmed that the live birth rate in poor responders after double embryo transfer was clinically and statistically significantly higher than after single embryo transfer with a modest increase in multiple birth rates.

In spite of apparent heterogeneity of the study population the major part of our patients were defined as poor responder and the oocyte accumulation gave the possibility to perform a multiple embryo transfer explaining so high clinical success rate in study's population. In fact 3 of 4 twin pregnancies occurred after transfer of 3 embryos derived from fresh and frozen oocytes.

In summary, our data demonstrate that transfer of embryos derived from vitrified natural oocytes and an embryo developed from the last fresh MNC assure an excellent clinical outcome compared to the repetitive single transfers following simple MNC. The study data also suggest that the natural oocyte accumulation by vitrification may increase the pregnancy rate of spontaneous IVF cycle to the levels obtained in protocols with ovarian stimulation. Although further and possibly randomized studies are necessary to confirm these results and draw any definitive conclusion regarding the role of natural oocyte accumulation, our preliminary results showed this strategy to be efficacious, safe, and minimally invasive alternative to stimulated cycles.

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