

# The Corelation Between Serum Estradiol ( $C_{23}H_{32}O_3$ ) Impact on Selected Biomarkers and Outcome of Fresh Embryo Transfer in Comparison with Freeze All Strategy *in vitro* Fertilisation Patients

DRAGOS ALBU<sup>1</sup>, ALICE ALBU<sup>1\*</sup>, ROMINA MARINA SIMA<sup>2</sup>,  
MIRCEA OCTAVIAN POENARU<sup>2</sup>, ADRIAN NEACSU<sup>2</sup>, OANA TOADER<sup>3</sup>,  
RADU CHICEA<sup>4</sup>, LIANA PLES<sup>2</sup>

<sup>1</sup> Carol Davila University of Medicine and Pharmacy, Maternal-Foetal and Reproductive Medicine Department, Medlife, 37 Dionisie Lupu Str., 020021, Bucharest, Romania

<sup>2</sup> Carol Davila University of Medicine and Pharmacy, Department Obstetrics Gynecology, Bucur Maternity, St. John Hospital, 37 Dionisie Lupu Str., 020021, Bucharest, Romania

<sup>3</sup> Carol Davila University of Medicine and Pharmacy, Department Obstetrics Gynecology, INSMC "Alessandrescu-Rusescu" 37 Dionisie Lupu Str., 020021, Bucharest, Romania

<sup>4</sup> Lucian Blaga University, Faculty of Medicine, Lucian Blaga. Str, 550169, Sibiu, Romania

**Abstract:** *In stimulated cycles the endometrium could be more advanced than the embryos, with a possible negative impact on their implantation capacity. Therefore, there is an ongoing debate regarding the best transfer strategy: freeze-all versus fresh embryos transfer. Our study aimed to analyse if the frozen only embryos-transfer strategy for in vitro fertilisation (IVF) has higher clinical pregnancy rate (CPR) than the traditionally fresh transfer. We performed a retrospective study in a private centre of reproductive medicine. We included only patients who performed fresh embryo transfers (n=245) and patients with all the embryos frozen and then transferred into a non-stimulated cycle (n=117). The reasons for delayed transfer were an increased risk of ovarian hyperstimulation syndrome or the increase of late follicular phase serum progesterone level. The mean age of the study group was  $34.33 \pm 4.28$  years and mean AMH was  $3.68 \pm 3.56$  ng/mL. Patients with fresh transfer were significantly older ( $p < 0.0001$ ) and obtained significantly lower number of oocytes at egg retrieval ( $p < 0.0001$ ) and lower zygotes number ( $p < 0.0001$ ) in comparison with patients with frozen transfer. After adjustment for confounders, CPR was significantly higher in patients with fresh transfer in comparison with those with frozen transfer in total group (OR 2.7,  $p = 0.001$ ) and in patients with cleavage stage embryo transfer (OR 6,  $p = 0.008$ ), but not in patients with blastocyst transfer. Similarly, the implantation rate was significantly higher in total group and in both subgroups ( $p < 0.0001$ ). Our study shows that the freeze all strategy should not be performed in all the patients, being inferior to fresh transfer in the total study group and cleavage stage embryo transfers. The decision to transfer frozen embryos should take into account the availability of blastocysts for transfer and the risk benefit profile.*

**Keywords:** CRYOTOP, IVF; implantation rate; fresh embryo transfer, clinical pregnancy rate

## 1. Introduction

In vitro fertilisation (IVF) is one of the most important achievements in medicine in the 20th century, but the success rate is considered still low, and scientists all over the world think permanently at different strategies to improve pregnancy rates. It is considered that controlled ovarian stimulation performed before IVF contributes to the advancement of the endometrium in comparison with the embryos, being one of the factors that negatively affect the IVF outcome by reduced implantation. Therefore, it was suggested that freezing all the embryos followed by their transfer in a subsequent, non-stimulated cycle, could be a useful strategy to improve IVF success. However, whether this approach is beneficial for all the patients or only for specific subcategories of patients is still debated.

\*email: [albualice@yahoo.com](mailto:albualice@yahoo.com)

All authors contributed equally to the manuscript

While some studies suggested that freeze all strategy could improve IVF outcome in all the patients [1, 2], a recent meta-analysis showed that live birth rate (LBR) is increased only in hyper-responders and in patients with preimplantation genetic testing for aneuploidy, but not in normal-responders [3]. On the other hand, a recent analysis of Society for Assisted Reproductive Technology (SART) registry showed that clinical pregnancy rate (CPR) and LBR are decreased in low and intermediate responders following freeze all strategy, being higher only in high responders [4]. Thus, current evidence does not strongly support the freeze-all strategy for all the patients, being even possible to harm reproductive outcome in patients who are not hyper-responders. Moreover, it was found that patients undergoing a freeze-all strategy are at increased risk for pre-eclampsia [3], suggesting that an adequate selection of patients which will benefit from freeze-all strategy should be performed to obtain an optimal risk-benefit profile. It was also showed that freeze all strategy is associated with a significant decrease of moderate/severe ovarian hyperstimulation syndrome (OHSS) [3], offering another potential benefit of this approach that should be taken into account. Therefore, defining the exact subcategory of patients that would benefit from fresh or frozen embryos transfers after a freeze all cycle is essential in IVF clinical practise to improve the treatment success. Since the different results of the studies regarding the success of freeze-all strategy over fresh embryo transfer could be also explained by the particularities of clinical practice in variate medical centres, we aimed to analyse the results of freeze-all strategy in terms of CPR and implantation rate in our reproductive medicine centre.

## 2. Materials and methods

We conducted a retrospective study in a private Reproductive Medicine Department on the IVF outcome related to the biochemical environment. The medical files of all the patients who underwent IVF between January 2017 and December 2017 were reviewed. Only patients who performed fresh embryo transfer (245 patients) or frozen embryo transfer after freeze-all strategy (117 patients) were included. In patients with a freeze-all strategy, all the embryos were frozen and then transferred into a non-stimulated cycle. The reasons for delayed transfers were increased risk of OHSS or the increase of late follicular phase serum progesterone level above 1.5 ng/mL. For the freezing-thawing process, we used vitrification by Cryotop method. The same culture media was used in all the patients during the study period. The frozen embryos were transferred on day 3 or 5 according to the American Society for Reproductive Medicine (ASRM) 2018 guidelines [5] three hours after warming. For preparing the endometrium in frozen transfers we started with the administration of a combined oral contraceptive for 12-21 days. After four days break, patients received estradiol valerate 4 mg/day for six days, then 6 mg/day for another six days. After 12 days of estradiol valerate administration, intravaginal progesterone was associated and continued until beta-HCG measurement showed no pregnancy or until 12 weeks of pregnancy. The cleaving embryos were transferred after three days of progesterone, while the blastocysts were transferred after five days of progesterone administration. All the patients included in the study were treated with long agonist protocol in association with a mixt gonadotropin stimulation protocol.

The risk of OHSS was considered increased in the presence of serum estradiol level above 5000 pg/ml, more than 20 oocytes retrieved at ovum pick up or moderate ascites and at least two of the following: maximum ovarian diameter above 100 mm, hematocrit above 45%, leucocytosis above 15 000/mm<sup>3</sup>, hydrothorax, dyspnea and oliguria.

Clinical pregnancy was considered to be present if foetal heartbeats were observed at a transvaginal ultrasound at 4 weeks after embryo transfer. Clinical pregnancy rate (CPR) was calculated as clinical pregnancy per embryo transfer. The implantation rate (IR) was calculated as the number of gestational sacs observed at transvaginal ultrasound 5-6 weeks after embryo transfer divided by the number of the transferred embryos.

Statistical analysis was performed with SPSS version 20. A p-value below 0.05 was considered indicative of statistical significance. Chi-square test was used to compare categorical variables between groups and the Mann-Whitney U test was used for continuous and discrete variables. Logistic

regression analysis was used to adjust for confounding variables when the dependent variable was a categorical one (clinical pregnancy) and multivariate linear regression for discrete dependent variables (implantation rate). Data are expressed as mean and standard deviations (SD) or percentages, as appropriate.

### 3. Results and discussions

A total of 362 patients were included in the study with mean age of  $34.33 \pm 4.28$  years and mean serum AMH of  $3.68 \pm 3.56$  ng/mL. Fresh embryo transfer was performed in 245 patients and freeze-all strategy followed by frozen embryo transfer was performed in 117 patients. Cleavage stage embryos were transferred in 201 patients, while blastocysts were transferred in 161 patients. Patients with fresh embryo transfer were significantly older in comparison with patients with frozen embryo transfer in total study group ( $34.9 \pm 4.1$  versus  $33.09 \pm 4.2$  years,  $p < 0.0001$ ) (Table 1), but the age of patients with fresh and frozen embryo transfer was similar in the subgroups of patients according to the transferred embryo stage (table 1). The number of the oocytes retrieved at egg collection ( $6.53 \pm 3.8$  versus  $13 \pm 5.9$ ,  $p < 0.0001$ ) and zygotes number ( $4.24 \pm 2.6$  versus  $8.3 \pm 4$ ,  $p < 0.0001$ ) was significantly lower in patients with fresh embryo transfer in the total study group and in subgroups according to day of embryo transfer (Table 1). A similar number of embryos were transferred in the two categories of patients ( $1.89 \pm 0.7$  in fresh transfers versus  $1.83 \pm 0.7$  in frozen transfers,  $p = \text{NS}$ ).

**Table 1.** Characteristics of the study group and subgroups of patients according to the day of embryo transfer

	Total (n=362)		Day 3		Day 5	
			n=201		n=161	
	Fresh n=245	Frozen n=117	Fresh (n=174)	Frozen (n=27)	Fresh n=71	Frozen N=90
Age (years)	$34.9 \pm 4.1^b$	$33.09 \pm 4.2$	$35.4 \pm 4.1^a$	$34.4 \pm 4.4$	$33.82 \pm 3.8^a$	$32.6 \pm 4.1$
Oocytes no	$6.53 \pm 3.8^b$	$13 \pm 5.9$	$5.1 \pm 2.9^b$	$9.48 \pm 3.7$	$10.1 \pm 3.2^b$	$14.1 \pm 6.1$
Zygotes no	$4.24 \pm 2.6^b$	$8.3 \pm 4$	$3.14 \pm 1.8^c$	$4.37 \pm 2.3$	$6.9 \pm 2.3^b$	$9.48 \pm 3.36$
IR	$0.29 \pm 0.39^c$	$0.19 \pm 0.32$	$0.23 \pm 0.33^c$	$0.07 \pm 0.22$	$0.45 \pm 0.47^b$	$0.23 \pm 0.34$
CPR	40.4% <sup>a</sup> (99/245)	29.9% (35/117)	37.4% <sup>b</sup> (65/174)	11.1% (3/27)	46.5% <sup>a</sup> (33/71)	35.6% (32/90)

CPR= clinical pregnancy rate, a = p value non-significant fresh vs frozen, b = p value  $< 0.0001$ , c = p value  $< 0.05$

In univariate analysis patients with fresh embryo transfer had significantly higher CPR in the subgroup with cleavage stage embryo transfer (37.4% versus 11.1%,  $p = 0.007$ ), but similar CPR in comparison with those with frozen embryo transfer in the total group (40.4% versus 29.9%,  $p = 0.053$ ) and in the subgroup with blastocyst transfer (46.5% versus 35.6%,  $p = \text{NS}$ ) (Table 1).

In logistic regression analysis, after adjustment for age, oocytes number, zygotes number and transferred embryos' number, fresh transfer was significantly associated with higher CPR in the entire group of patients (OR=2.7,  $p = 0.001$ ), in patients with cleavage stage embryo transfer (OR=6,  $p = 0.008$ ), but not in those with blastocyst transfer (OR=2,  $p = 0.066$ ) (Table 2).

**Table 2.** The association between fresh embryo transfer and clinical pregnancy and implantation rate after adjustment for confounders

	OR for CPR of fresh transfer*	<i>p</i> value	Beta value for IR of fresh transfer**	<i>p</i> value
Total study group	2.7	0.001	0.469	<0.0001
Cleavage stage	6	0.008	0.689	<0.0001
Blastocyst	2	NS	0.416	<0.0001

\*in logistic regression with CP as dependent variable and age, oocytes number, zygotes number and transferred embryos number as independent variables; OR=odd ratio

\*\*in multiple linear regression with IR as dependent variable and age, oocytes number, zygotes number and transferred embryos number as independent variables

Implantation rate (IR) was significantly lower in patients with frozen embryo transfer ( $0.19 \pm 0.32$ ) in comparison with patients with fresh embryo transfer ( $0.29 \pm 0.39$ ,  $p < 0.01$ ) in the entire study group and in subgroups according to embryo's stage:  $0.07 \pm 0.22$  versus  $0.24 \pm 0.33$  in cleavage stage embryos transfer ( $p = 0.005$ ) and  $0.23 \pm 0.34$  versus  $0.46 \pm 0.47$  in blastocyst stage transfer ( $p = 0.003$ ).

Fresh embryo transfer was independently and positively associated with implantation rate after adjustment for confounders (age, oocytes, zygotes and transferred embryos number) in the entire study group (beta=0.469,  $p < 0.0001$ ) and in the subgroup with cleavage stage embryo transfer (beta=0.689,  $p < 0.0001$ ) and blastocyst transfer (beta=0.416,  $p < 0.0001$ ) (Table 2).

In patients with frozen embryo transfer the presence of clinical pregnancy was associated with blastocyst transfer (OR 12,  $p = 0.004$ ) and with higher number of oocytes obtained at egg collection (beta=0.101,  $p = 0.043$ ) after adjustment for age, zygotes number and transferred embryos number.

Our study shows that, in our daily clinical practice, transfer of frozen embryos in a freeze-all strategy is not appropriate for all patients undergoing IVF, the CPR and IR being lower than in patients with fresh embryo transfer. In particular, the subgroup of patients with day 3 embryo transfer had lower CPR when frozen embryos were transferred, suggesting that in these patients freeze all strategy should be avoided.

An aspect that should be taken into consideration is the retrospective design of our study. Therefore, in contrast with a randomized controlled study which would randomly assign patients with similar clinical characteristics and reproductive prognostic to fresh or frozen embryo transfer after freeze-all strategy, in this study we just analyzed the reproductive outcome in patients with different reproductive behaviour. Thus, patients with the freeze-all strategy were selected based on their increased risk for ovarian hyperstimulation syndrome or the increase of late follicular phase serum progesterone. Therefore, there is an increased probability that some of these patients to have polycystic ovary syndrome which represents a category of patients with a good ovarian reserve and without any convincing data regarding decreased quality of oocytes or embryos. Similarly, patients with an increase of late follicular phase serum progesterone are a category of patients without a demonstrated decrease of embryos quality [6,7]. Taking into account the higher number of oocytes in patients performing freeze all, most of these patients have good or even high ovarian reserve with a good reproductive prognosis. Even so, the CPR and IR were lower in these patients who performed frozen embryo transfer after freeze all, suggesting that the transfer of frozen embryo negatively affects the chance of pregnancy. Since the endometrium is probably better in unstimulated cycles as demonstrated by several authors [8-10], we can assume that the limiting factor for IVF success is the embryo competence.

Whether the human embryo's competence is affected by cryopreservation is mostly unknown. There is a limited number of studies performed on human embryos for ethical reasons [11]. However, experimental studies on animal embryos showed that cryopreservation could lead to embryonic cells and DNA damage [11-13] and could affect the expression of various genes [14,15]. Moreover, the



activity of the cytoplasmic enzyme seems to be affected by cell rupture produced by ice crystals during vitrification [16]. Although there is a theoretical basis for a negative impact of cryopreservation on embryos competence, the data regarding the implantation rate and pregnancy rate of frozen embryos are divergent. Thus, while Roque et al. found a significant increase in live birth rate with frozen embryo transfer compared with fresh embryo transfer [2], the study of Acharya et al. [4] reported higher CPR and LBR with frozen embryo transfer only in high responders and lower CPR and LBR in intermediate and low responders. Based on the results of Acharya et al. [4] we can conclude that cryopreservation could be detrimental to embryo competence in subcategories of patients like intermediate and low responders. In our study, the detrimental effect of cryopreservation was shown in patients with day 3 embryo transfer, suggesting that these embryos were more susceptible to freeze related injuries. Taking into account the Department's strategy to transfer cleavage stage embryos when there are little chances that the embryos will develop further, we can assume that patients with cleavage stage transfer had embryos with lower embryos quality. Consequently, we can hypothesize that these embryos could have decreased defence mechanisms against injuries, explaining their reduced IR and lower CPR compared with fresh embryo transfer.

In patients with frozen embryo transfer, the transfer of blastocyst was an independent predictor of clinical pregnancy after adjustment for confounders, supporting the superiority of blastocyst transfer for IVF success in frozen cycles. The data in the literature do not uniformly support our results, a recent meta-analysis showing that CPR is similar in transfers of frozen blastocysts or cleavage-stage embryos [17]. In turn, other authors showed that frozen blastocyst transfer is superior to cleavage-stage embryo transfer in both poor and normal responders [18].

There are some clinical implications of our study. Thus, when we decide to apply the freeze-all strategy for an increased OHSS risk we should take into consideration the availability of blastocysts for transfer to avoid a decrease of pregnancy chances. On the other hand, in patients with high late follicular phase serum progesterone, when the aim is to increase the chances of pregnancy, we should weight carefully the risk-benefit profile.

#### 4. Conclusions

Our study shows that the freeze-all strategy is not superior to the transfer of fresh embryos and the decision to freeze all the embryos for transfer in a subsequent cycle should be carefully weighed in each patient based on patient profile and possible benefits. While the transfer of a good quality blastocyst to avoid OHSS in a patient with a high risk for such complication and with good chances for high cumulative pregnancy rate with subsequent frozen embryos transfers could be justified, in patients with poor prognosis and high late follicular phase serum progesterone this strategy should be carefully weighed.

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