# Implantation and pregnancy rates from vitrified embryos

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## ABSTRACT

**Objective:** The main objective of this study was to evaluate the effectiveness of vitrification technique through embryo viability, embryo implantation rate and clinical pregnancy in patients undergoing controlled ovarian stimulation.
**Material and methods:** Viable embryos of patients deriving from *in vitro* fertilization were vitrified using the protocol of Irvine Scientific by closed system. 28 cycles of freezing and thawing of embryos were evaluated during the periods from January 2010 to December 2012 in the Clinical Reproduction, Presidente Prudente – SP.
**Results:** Total of 176 embryos was frozen. The average number of frozen/thawed embryos per patient was 5, totaling 140 thawed embryos. These thawed embryos, 78,6% (110/140) displayed viable morphologically (at least 50% from intact blastomeres) and 75% (105/140) of thawed embryos were transferred. In 28 cycles in which there was transference, an average of 3.75 embryos were transferred per patient, from which resulted in 17 biochemical pregnancies and of these 8 pregnancies reached term (28,6%) and embryonic implantation rate was 20%.
**Conclusion:** It can be concluded that the method of embryos vitrification is an effective technique, with high survival rate, allowing the storage of surplus embryos with satisfactory pregnancy rates in thawing cycles.
**Key Words:** vitrification, embryos, implantation, cleavage.

## RESUMO

**Objetivo:** O objetivo principal deste estudo foi avaliar a eficácia da técnica de vitrificação na viabilidade dos embriões, implantação e gestação com intuito de introduzir como procedimento de rotina em pacientes com hiper-estímulo ovariano.
**Materiais e Métodos**: Embriões viáveis de pacientes provenientes de fertilização *in vitro* foram vitrificados utilizando o protocolo da Irvine Scientific pelo sistema fechado. Foram avaliados 28 ciclos de vitrificação e desvitrificação de embriões realizados entre os períodos de janeiro de 2010 a dezembro de 2012 na Clínica Reproduction, Presidente Prudente – SP.
**Resultados:** Foram vitrificados no total de 176 embriões. O número médio de embriões vitrificados/desvitrificados por paciente foi de 5, totalizando 140 embriões desvitrificados. Desses embriões desvitrificados, 78,6% (110/140) apresentaram-se morfologicamente viáveis (pelo menos 50% dos blastômeros íntegros) e 75% (105/140) dos embriões descongelados foram transferidos. Nos 28 ciclos em que houve transferência, uma média de 3.75 embriões foram transferidos por paciente, dos quais resultaram em 17 gestações bioquímicas, e destes 8 gestações chegaram a termo (28,6%) e taxa de implantação embrionária foi de 20%.
**Conclusão:** Pode-se concluir que o método de vitrificação de embriões é uma técnica eficiente, com altas taxas de sobrevida, possibilitando o armazenamento dos embriões excedentes com boas taxas de gravidez em ciclo de descongelamento.
**Palavras-chave**: vitrificação, embrião, implantação, clivagem.

## INTRODUCTION

The embryonic cryopreservation is a technique widely used in assisted reproduction clinics whose primary indication consists in storage of surplus embryos obtained through of a cycle of *in vitro* fertilization (IVF) that were not transferred, allowing a new attempt at pregnancy. Situations such as in the case of a patient who develop ovarian hyperstimulation (SHO), or in the case of risk of loss of reproductive function such as, for example, before of chemotherapy treatment, the freezing of all the embryos is performed, without implementation of the transfer of fresh embryos (Michelmann & Nayudu, 2006; Bedoschi & Oktay, 2013). Then, the freezing of embryos allows an improvement and an increase in safety of assisted reproduction techniques becoming an extremely important therapeutic strategy at assisted reproduction clinics.
The vitrification is a technique of ultra-fast freezing whose principle of the technique relies on occasions to rapid dehydration of the cells using high concentrations of cryoprotectants, followed of a rapid freezing in liquid nitrogen causing them to go into a solidified state thereby preventing formation of intracellular ice crystals, extremely beneficial for the survival of cells (Liebermann and Tucker, 2006; Kasai, 2004; Fuller and Paynter, 2004). This method is increasing its popularity it has been shown to be a fast, efficient, secure and lowest cost for the cryopreservation of embryos at any stage of development (cleavage until blastocyst) than the slow freezing technique. So that the result of vitrification and thawing is efficient is important take into account the speed of freezing, concentration of cryoprotectants, volume of solution and embryo quality.
The embryos can be frozen on days 1 to 6 development. The survival rate of freezing and thawing process is around 75% being the embryonic unviable post thawing related to the loss of blastomeres in this process. Is highly probable that those embryos that do not survive the defrosted are the same that not to have reached the embryonic development through deployment. The pregnancy rate depends on the age of the woman, the number and quality of frozen embryos may reach 20% per embryo transferred (Oktay & Sonmerzer 2007; Sonmezer & Oktay, 2004). And a recent study related that women who have had fertility treatment using frozen embryos had greater chance of success compared with those who did fresh embryos.
A review of over 37.000 pregnancies from *in vitro* fertilization showed that there was a reduced chance of mothers goes through miscarriage and premature births and low birth weight babies when the embryos were stored and frozen compared with treatment involving fresh embryos transfer (Maheshwari *et al.,* 2012). For these researchers there may be a difference in uterine environment between fresh cycles, when the embryos are transferred soon after the oocytes have being collected, in compare with frozen cycles, when the utero not was stimulated on days before of transference (Maheshwari & Bhattacharya, 2013).
In 2008, Danish scientists also found that babies born from frozen embryos had more weight. They attributed the fact that the top quality of the embryos survived the freezing process.
The routine in most IVF clinics is that the best embryos are transferred first to fresh and only the surplus of good quality embryos is frozen for transfer at a later stage. If pregnancy rates are equal and the result in birth of babies are better maybe it should take into account the possibility of freezing all the embryos and transfer them at a later period than do the transfer in fresh.

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## OBJECTIVE

Establish as routine an efficient vitrification method for preserving embryos allowing high survival rate after thawing and implantation.

## MATERIALS AND METHODS

The study was conducted at Reproduction Clinic, in Presidente Prudente, on January 2010 to December 2012. All patients underwent ovarian stimulation with recombinant FSH (Gonal-F, Serono, Geneva, Switzerland), after pituitary suppression with GnRH agonist. When at least 2 follicles reached18mm diameter was administered hCG for final follicles maturation, recombinant hCG – 250ug (Ovidrel, Serno, Geneva, Switzerland). The oocytes collection was performed vaginally, 35 to 36 hours after ultrasound--guided. The obtained oocytes were incubated for 4 hours in culture medium (G1Plus, Vitrolife, Switzerland), afterwards denuded and evaluated the degree of maturation and the ICSI (intra-cytoplasmic sperm injection) was realized in those classified in metaphase II, according to technique described by Palermo *et al* (1992).

The embryonic culture was performed in microdroplets 50ul of G1Plus medium (Vitrolife, Switzerland) and covered with paraffin oil in a humidified atmosphere under 6% $CO_2$ in air at 37ºC. Selected for transference or cryopreserved embryos were evaluated according to the classification system of Veeck (Hsu *et al.,* 1999).

Viable embryos that were not transferred, they were vitrified in blastomeres stage, in third day of culture, using the protocol of Irvine Scientific vitrification (Cryopreservation Protocols, 2006) with closed system. Embryos showing multinucleation, cleavage stoppage or more than 50% of fragmentation were not vitrified.

During the study period, 28 cycles of vitrification/thawing from embryos D+3 were realized, which resulted in 28 transfers.

### Vitrification

The technique consisted of a series of 2 solutions primarily composed by buffered culture medium with Hepes supplemented with Substitute Synthetic Serum (Irvine Scientific, EUA) with Glycol Ethylene (EG) and Dimethyl Sulfoxide (DMSO) permeable cryoprotectants in increasing concentrations to environment temperature.

Initial 1 or 2 embryos were transferred into 20ul droplet of equilibrium solution ES (Irvine Scientific, EUA) that have 7,5% Glycol Ethylene (EG) and 7,5% Dimethyl Sulfoxide (DMSO) in buffered medium with Hepes supplemented with 20% Substitute Synthetic Serum (SSS) at environment temperature for 5 to 15 minutes. After this time, the embryos were transferred, with possible minor volume of ES, consecutively in three droplet 20uL of solution of vitrification VS1, VS2, VS3 remaining for 5 seconds on each droplet at environment temperature. The time that the embryos remained in the solution VS (15% Glycol Ethylene and 15% Dimethyl Sulfoxide and sucrose 0,5M in buffered medium with supplemented with 20% SSS) until the immersion of cryotip pallet (Irvine Scientific, EUA) in liquid nitrogen (LN2) not must exceed 90 seconds. The embryos were deposited in cryotip pallet, with minimal volume of vitrification solution, sealed the two tails ends and then immediately immersed in liquid nitrogen. To secure stockpiling the cryotip pallet was stored in a closed case with identification.

### Thawing

The pallets to be thawed were selected in tank of liquid nitrogen and transferred to the tank filled with LN2. For heating of embryos, the cryotip pallet was removed from liquid nitrogen and quickly submerged in water bath at 37ºC for 3 seconds. The cryotip pallet was dried with sterile gazes, afterwards was cut the extremity where it will be connected to the syringe and carefully cut the other extremity and ejected the embryos in thawing solution increasingly reduced concentration of sucrose. The embryos were first transferred in heating solution containing sucrose 1M (TS, Irvine Scientific, EUA) diluted in buffered medium with 20% SSS at environment temperature for 1 minute. Then transferred to solution dissolvent of sucrose 0,5M (DS, Irvine Scientific, EUA), dissolved in buffered medium with 20% SSS, by 2 times, 2 minutes each at environment temperature, and then washed in buffered solution (WS, Irvine Scientific, EUA) composed only of buffered medium with 20% SSS by 3 times, 3 minutes each.

After heating of embryos, the evaluation of survival was performed using the morphological criteria. The survival of embryos was defined by re-expansion of the same, having maintained intact cellular membrane, normal ooplasm and pellucid zone, as well as perivitelinic space of normal size.

After removal of crioprotectants, the embryos were washed and cultivated in 50ul droplet of medium G2Plus (Vitrolife, Switzerland) under paraffin oil and maintained in incubator under 6% CO2 in air at 37ºC by a interval of 2 to 4 hours. The embryos were re-evaluated by classification and then after signing the consent form of the number of embryos to transfer, embryo transfer at least 3 viable embryos was performed. Only embryos with at least 50% of intact blastomeres after thawing were selected to transfer. None embryos was submitted the technique of "assisted hatching" and "ICSI with high magnification".

The endometrial preparation for transfer was prepared with daily dose of 6mg of estradiol orally (Estrofem, Medley) from the first day of menstruation. The endometrial thickness and morphology were analyzed over a period of at least 12 days of administration of estradiol considered appropriate endometrial thickness exceeding 7mm. Progesterone (Ultrogestan, Farmoquímica) was administered by vaginal via in dose of 900mg per day. The embryo transfer was performed in the third or forth day of progesterone under ultrasonographic monitoring using catheter Wallace PEB 683. Past fourteen days of the transfer was prompted examination of serum B-hCG quantitative. In fourth week after transfer clinical pregnancy was diagnosed by the presence of embryo with heartbeat ultrasound.

## RESULTS

During the study period 28 cycles of vitrification/thawing of embryos were performed with the mean age of women was 32,1+3,38. Total of 176 embryos were frozen for which afterward were devitrified 140. The survival rate of vitrified and devitrified embryos was 78,6% (at least 50% of integrity blastomeres) and 75% of thawed embryos were transferred. The average number of vitrified/devitrified embryos per patient was 5.0+5,39 (varying of 1 to 9 embryos). 28 transferences with total of 105 embryos were realized, an average of 3.75 embryos per patient.

The global rate of biochemical pregnancy was 60,7% (17/28), however, the pregnancy rate that reached term was 28,6% (8 pregnancies in 28 cycles of thawing with transference) and implantation rate was 20,9% by thawing cycle. 8 of term pregnancies, 62,5% (6) was of single pregnancy and 37,5 (3) of twin pregnancy.

## DISCUSSION

The proposed method of vitrification and studied seems to have demonstrated as an efficient method and offers the possibility of embryos cryopreservation with extreme efficiency. This freezing method demonstrates some advantages, especially avoiding the formation of ice crystals and the cellular injury occurred in slow freezing (Watson & Morris, 1987). These events injure essentially the cytoskeleton and the cellular membrane (Pickering et al., 1990, Ghetler et al., 2005), avoided by rapid freezing. The simplicity of the method, and not need for sophisticated equipment considerably reduces cost of the whose procedure and particularly avoids bear potential risks to patient without the need for a new cycle of ovarian stimulation, such as exposure to anesthesia or the development of ovarian hyperstimulation syndrome.

In contrast it presents some disadvantages, since use high concentration of cryoprotectants that are toxic to cells.

Seeking to minimize this toxicity, several combinations of some cryoprotectants agents during the vitrification process are being studied and researched, specially the Glycol Ethylene, DMSO, Glycerol, 1,2-propylene glycol and sucrose (Wrigh et al., 2004; Shaw et al., 2000).

There is a great variation between the vitrification protocols being the most significant difference is in type of physical support (cryotip, cryotop, OPS, Cryoloop, HSV, etc), however the good result of technique, independent of methodology applied, depends on embryo quality, the exposure time of sample in cryoprotectant, as well as reduced volume of solution in witch the sample should be stored. It was established in service using the closed system, cryotip (Irvine Scientific), by presenting issues of contamination from direct contact of embryos and liquid nitrogen (Kuwayama et al., 2005).

After the transfer, the surplus embryos were vitrified, with satisfactory morphological characteristics, namely, integral pellucid zone, shared with at least 6 cells in day D+3, that presented symmetrical blastomeres, or with little asymmetry and with at least 50% of fragmentation.

In this study the survival rate after the thawing of embryos was 78,6% (110/140). The rate of biochemical pregnancy, pregnancy to term and implantation of vitrified embryos on the third day was of 60,7% (17/28), 28,6% (8/28) and 20,9% (6/28) respectively. In neither cases was submitted the technique of "assisted hatching" (aperture of pellucid zone of embryo) or "ICSI with high magnification". Selmo Geber at Origen Clinic (2005) achieved a indices of pregnancy approximately 30% originating from frozen embryos, but has been made the use of technology by laser that allow to do an orifice in pellucid zone of embryo, which according the author, it would facilitate the your implantation, since the freezing could to take to a increase of thickness of pellucid zone, what would reduce the possibility of implantation.

Desai and collaborators (2010) showed clinical and obstetrics outcomes of vitrified embryos transfer in day D+3 in cleavage stage. A total of 270 transferences were performed and the clinical pregnancy and the implantation rates in patients under of 38 years (n=200) were 45% and 24%, respectively. In case of women above of 38 years, there was decline of 29% and 13% (n=70).

The thawing resulted in embryos with alive and dead blastomeres, what is shown as a disadvantage of this process, since the evaluation of viability becomes difficult. In general, an embryo that has more of 50% of viable blastomeres viable in thawing is considered a survivor. There is not convincing evidence that the loss of one or two blastomeres is clearly harmful to the embryos in your initial development. Although it has been reported that embryos entirely intact show a higher implantation rate than those partially intact.

The development of more effective cryopreservation techniques can extend the currently existing indications. In assisted fertilization cycles, large part of the fresh embryo implantation failures can be attributed to endometrial, which tend be advanced histologically in relation to the development of embryo due to high hormonal levels attained in an induced cycle. One way to improve the synchronization between the endometrial histology and the development of embryo would be cryopreserve all the viable embryos produced in an IVF cycle and transfer them later after endometrial adequate preparation. Other dare in assisted reproduction is the attempt of minimizing the incidence of multiples pregnancies due to transfer more than one embryo obtained after the IVF treatment. Currently the best approach to try to avoid multiple births is the transfer of a single embryo and for it is necessary to extend the embryo culture to the blastocyst stage. Usually, the embryo transfer occurs at the stage of cleavage when they are with 8 cellules. But the genome of the embryo is activated after the third day of development. So when postponed the embryonic transference to fifth day, where it reaches the blastocyst stage, there is a better evaluation and transfer of a minor number of embryos. However, the option to defer the transfer to blastocyst stage depends on a series of laboratorial factors, inclusive of affording a good cryopreservation technique. Patients who are considered candidates for cultivation of blastocyst must be younger than 35 years old with a good ovarian reserve or patients of advanced age with an adequate number of pro-nuclear embryos. The number of embryos observed on the day 3 and the ability of embryonic cleavage are also important criteria for selecting the appropriate cases for this procedure.

The main point in getting good results with vitrification is the apparatus used which it must allow the possibility of vitrificating the embryos with minimal volume of cryoprotectant possible and also allow the procedure in the shortest possible time. The use of closed system criotip eliminates the potential of oocytes/embryos contamination during the cryopreservation and storage, without compromising the survival and development rates in vitro. However, it's very important that the embryologist must be careful, the expert and the dexterity during the stages of exposition of biological material in solutions containing high concentrations of cryoprotectants. Minor variations of a few seconds of exposure besides of adequate, especially in the last stage VS can affect the viability of the material and be major cause of poor results achieved in many laboratories, in term of survival of embryo, your potential of fertilization, implantation and pregnancy. It is not advisable freeze more than 2 embryos by time, since the manipulation must be very precise and realized in specific times.

The technique of vitrification of embryos described in the present study showed good survival results and implantation after thawing and transfer reaching a pregnancy indices 28,6%, next to from Latin-American that is 30%. However, it is necessary more studies and more samples to confirm the efficiency of this technique of vitrification.

Currently in the service is performed "assisted hatching" in all vitrified embryos D3. It was established that in cases of in vitro fertilization in that there is minimal number of viable embryos with less 20% of fragmentation the embryonic cultivo is prolonged until the fifth day, with the intuit to reduce the number of embryos transferred and keep good rates of pregnancies, besides reduces the rate of multiple pregnancy. Case to have blastocyst surplus is vitrified following the blastocyst vitrification protocol from Irvine Scientific (EUA). This will permit a better selection of embryos to be preserved and transferred in future and we believe that with these modifications it will has a significant increase of pregnancy at term and reductions in rate of spontaneous abortion and in the number of surplus embryos.

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