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Cryopreservation of incomplete compacted morulae and preliminary biopsy of excluded fragments

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Running title: Incomplete compacted morula cryopreservation

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Abstract

The complexity of predicting the embryo development potential at the cleavage stages and emergence of epigenetic risks during prolonged *in vitro* cultivation of pre-implantation embryos are advantageous when transferring the embryo at the morula stage to the uterine cavity. However, the criteria for embryos estimating at this stage which allow predicting the cryopreservation outcomes have been poorly described. All day 4 embryos (n=224) were graded 1, 2, 3, 4 or 5 according to blastomere compaction degree (BCD= 100%, 75%, 50%, 25%, 0%, respectively) and then the survival and blastocyst formation rate of these morulae were studied after cryopreservation. An inverse dependence was found between the survival rate and BCD. Excluded fragments were characterized by low osmotic reaction during exposure with cryoprotective media and after freeze-thawing they have been destroyed. As damaged necrotic areas of the embryo can affect their further development rate we proposed blastomeres and excluded fragments biopsy of incomplete compacted morula prior to the embryo cryopreservation. This led to significant increase of their post-thawing survival rate up to (93.1 ± 4.1), (75 ± 8.8) % and blastocyst formation rate up to (85.2 ± 10.4), (59.4 ± 5.2)% in grades 2 and 3 respectively, and there was no significant difference in grade 4. Thereby using the blastomeres and excluded fragments biopsy in incomplete compacted morulae can improve the cryopreservation outcomes of embryos with BCD grade 2 and 3.

Key words: cryopreservation, morula, biopsy, incomplete, blastomere compaction degree.

Introduction

Cryopreservation of gametes and embryos has become an important biotechnological component of infertility treatment cycles by assisted

reproductive technologies (ART). Nowadays the embryos are successfully cryopreserved using all the stages from the fertilized oocytes to hatched blastocyst. Cryopreservation can be achieved by either controlled rate slow cooling or vitrification (Rienzi *et al*., 2017; Edgar & Gook, 2012). However, each embryonic stage has its own morphological features which need to be taken into account during cryopreservation (Rienzi *et al*., 2017). Embryos cryopreservation at the pronuclear stage makes it possible to obtain a large number of embryos to transfer for a subsequent cryo cycle treatment, but it is quite difficult at this stage to predict after thawing the quality of embryo, development and kinetic potential during progression to more advanced stages. In addition, the effectiveness of embryo cryopreservation at the zygote stage depends on the phase of cell cycle, since the microtubules of the mitotic spindle are highly susceptible to cryopreservation factors (Balakier *et al.*, 1993).

Up to days 2 and 3 of development the embryos consist of 2-8 blastomeres. However, at this stage there is a possibility of either lysis of individual blastomeres or an increased fragmentation degree that does not allow to consider this stage as optimal for freezing.

Day 5 embryos as a rule reach the blastocyst stage and their concurrent morphology enables the evaluation of the prospects of implantation and further intrauterine development rates of the embryo (Van den Abbeel *et al*., 2013; Irani *et al.*, 2017). However, the features of this embryo stage, namely the existence of a large fluid filled cavity (blastocoel) in the blastocyst cavity, may require a longer exposure time with cryoprotective agents (CPA) solution, thereby increasing the risk of potential CPA toxic effects for the embryo; in addition it has been suggested that the presence of the large water content in embryo cavity increases the risk of ice crystal damage during freeze-thawing. In this regard, blastocoel collapsing procedures are performed to remove excess water inside, which requires additional embryological manipulations (Kovačič *et al.,* 2018).

It is therefore an opportunity to separately consider the day 4 development stage of human embryo, i.e. morula. Exactly at this stage of *in vivo* pre-implantation development embryos enter to the uterine cavity during natural conception. In addition, there is an increase in the number of blastomeres and a decrease in their individual cell volumes as a result of the embryo cell division. Segregation of blastomeres towards either the center or the periphery of the cell mass, making tight intercellular junctions characterize collectively the development of the offensive morula stage (Fabozzi *et al.*, 2016). These structural features of this embryo development stage make it possible to increase the blastomere permeation rates with cryoprotective solutions and to reduce the exposure time, thereby it renders less cytotoxic effect of cryoprotectants. At the same time, the individual morphological characteristics of morula contribute to their further development and implantation rates. Thus, the large number of fragmentations reduces the size of the intracellular mass and trophectoderm cells (Ivec *et al*., 2011). As well the embryos with incomplete compaction have a reduced blastocyst formation rate and implantation potential (Ebner *et al*., 2009).

Materials and Methods

In this retrospective study we have compared the survival rate of incomplete compacted morulae. The study included 224 morulae cryopreservation cycles performed since January 2015 to September 2017.

Ovarian stimulation

Human gametes and pre-implantation embryos were obtained in the cycles of infertility treatment using IVF. The mean age of women in the ICSI cycles was 30.3±4.2 years. Stimulation was performed in a standard manner using a luteal long protocol of gonadotropin-releasing hormone (GnRH) agonist (Decapeptyl, Ferring, Malmo, Sweden) or GnRH antagonist (Cetrotide, Serono, Geneva, Switzerland) with daily administration of recombinant follicle-stimulating

hormone (FSH) (GONAL-f, Serono). Oocytes were retrieved by transvaginal aspiration under ultrasound guidance in 35 hours after the injection of recombinant human chorionic gonadotropin (Ovidrel, Serono).

IVF and ICSI

The retrieved oocytes were briefly exposed to 80 IU/mL hyaluronidase (Sydney IVF Hyaluronidase, Cook Medical, Spencer, IA, USA), and mechanically separated from their surrounding cumulus cells by aspiration through a glass pipette. All the oocytes at M II stage were selected for micromanipulation.

The ICSI procedure was based on the protocol (Palermo *et al*., 1992). Fertilization was assessed at 16-20 hours after insemination by the presence of two pronuclei and two polar bodies. Normally fertilized oocytes were transferred into 0.8 ml culture medium Global total (Life Global, USA).

Grading of morulae compaction

Blastomere compaction of embryos to day 4 was evaluated and graded. The processing of the embryo images obtained by means of microscope Olympus IX-71 (Olympus, Japan) was carried out using a program Axio Vision. The shape of the circled areas of the embryo compacted and uncompacted parts were approximated as a sphere or ellipsoid. Calculating their relative volumes determined the degree of blastomeres compaction.

BCD (blastomere compaction degree) $= \frac{V1}{V2} \times 100$;

$$\text{BCD} = \frac{\frac{1}{6}\pi {D_1}^3}{\frac{1}{6}\pi {D_2}^3} \times 100 = \frac{{D_1}^3}{{D_2}^3} \times 100(\%)$$

V_1– volume of embryo compacted area;

D_1 – diameter of embryo compacted area;

V_2 – volume of whole embryo blastomeres;

D_1 – diameter of whole embryo blastomeres.

According to the BCD all the morulae (n=224) were divided into five groups: grade 1 – full compaction (BCD is 100%, n= 37), 2–4 grades – the compaction occupies not whole embryo (BCD were about 75, 50, 25 %, n = 52, 55, 51 respectively) and 5 grade – embryo at morula stage of development with no compaction (n = 29) (Fig. 1).

Every morula was analyzed by two embryologists independently. They made images of morula by themself and calculated the BCD index and then compared the results. 97% (224/218) of results we matched.

Figure 1 Grade of blastomere compaction degree. 1 – full compaction; 2–4 – a compaction occupies 75, 50, 25% of embryo volume, respectively; 5 – morula with no compaction.

Cryopreservation and thawing protocols

Cryopreservation was achieved by vitrification. The vitrification procedure was performed by the Cryotop method (Kuwayama *et al*., 2005). Morulae were equilibrated in 7.5% (v/v) ethylene glycol (EG) + 7.5% dimethyl sulfoxide (DMSO) in Global total medium at 20 °C temperature for 15 minutes. Then they were placed for 1 min into vitrification solution, consisting of 15% ethylene glycol + 15% DMSO + 0.5 M sucrose in the same medium as equilibration solution. Afterwards the embryos were placed on the Cryotop strip immediately and plunged into liquid nitrogen. For warming, the Cryotop was removed from liquid nitrogen and instantly placed in 1.0 M sucrose in Global total medium at

37°C for 1 minute and then were placed in 0.5 M sucrose in Global total medium for 3 minutes; after that they were placed into this medium for further culturing.

After warming the survival rate and developmental kinetics of embryos were evaluated by daily observations of morulae with grade 1.

Biopsy of excluded fragments in embryos with incomplete compaction

The excluded fragments were removed using the Olympus inverted microscope equipped with Hoffman system and Narishiga hydraulic micromanipulators (Narishiga, Japan) 1.48 mm wavelength diode laser (OCTAX Laser Shot TM System, Germany) in a computer-controlled contact mode. This was used for *Zona pellucida* drilling (12-13), with one blunted pipette about 12–17 μm, excluded fragments were removed. Embryos were washed with HTF medium containing 10% SSS and incubated at 37 °C with 5 % CO_2 until the time of transfer (Fig. 2). Removal of excluded fragments were carried out for morulae grade 2 (n= 18), grade 3 (n=19) and grade 4 (n=18) .

Figure 2 Excluded fragments removal procedure. Embryo development to blastocyst stage, magnification x600, (n=55).

Data analysis

Statistical analysis of the data was carried out using the Past 3.0 software. The quantitative data distribution was evaluated using the Shapiro-Wilk test. Results are presented as the mean ± standard deviation. For comparison of two independent samples, a nonparametric Mann-Whitney test was used, for paired comparisons were applied the Wilcoxon signed rank test. Differences were considered significant at $p < 0.05$.

Results

The survival rate of embryos after incubation stage in vitrification solutions for categories 1 and 2 was 100%, and for categories 3–5 they were (87 ± 7.4), (68 ± 5.9) and (53 ± 6.7)% respectively. We have noted the different osmotic reactions of compacted and non-compacted morulae parts at the incubation stage. Thus, the compacted part responds to the change in osmotic pressure as a single whole, in contrast to the regions not involved into embryo compaction. After embryo vitrification and warming the survival rate of category 1 was (98 ± 6.2) %, for categories 2–5 were (85 ± 4.1), (38.2 ± 4.4), (50 ± 5.8) and (22.2 ± 4.4) % respectively (Fig. 3).

Figure 3 Survival rate of morulae with different grade of compaction after vitrification. A statistically significant difference was observed between vitrified morulae and biopsied morulae with the same grade of compaction marked with an asterisk (*) and between vitrified grade 1 morulae and the study group – (#), ($P<0.05$).

The blastocyst formation rate (BFR) of morulae correlated with their survival rate after freeze-warming ($r = 0.94$). The cleavage rate of embryos to the blastocyst stage was (92.8 ± 7.9), (71.3 ± 8.2), (44.7 ± 9.9), (12.4 ± 4.9) % for categories 1–4 respectively(Fig. 4). There were no blastocysts in group with grade 5 morulae either fresh or vitrified.

Figure 4 Blastocyst formation rate of morulae with different grade of compaction after vitrification. A statistically significant difference was observed between fresh morulae and vitrified morulae or biopsied morulae with the same grade of compaction marked with an asterisk (*) and between grade 1 fresh morulae and the study group – (#), ($P<0.05$).

Damaged necrotic areas of the embryo can affect their further development rate and viability as a result of the release of toxic metabolites or inhibitory cell molecular signaling pathways (Van den Abbee *et al*., 1997).

We suggested that the removing of uncompacted fragments in grades 2-4 morulae by biopsy prior cryopreservation will reduce the negative effect of necrotic factors and thereby increase their survival and blastocyst formation rates. We did not biopsy the grade 5 morulae because of the compaction absence.

Removal of blastomeres and excluded fragments of incomplete compacted morula prior to the embryo cryopreservation led to an increase of their post-thawing survival rate up to (93.1 ± 4.1) and (75 ± 8.8)% and blastocyst formation rate up to (85.2 ± 10.4), (59.4 ± 5.2)%, $p<0.05$ in categories 2 and 3 respectively (Fig. 3, Fig. 4).There was noted an increase in blastocyst formation rate after using this technique for grade 4 morulae prior cryopreservation in contrast to such affect to their survival rate.

Discussion

The complexity of predicting the embryo development potential at cleavage stage and emergence of epigenetic risks during the prolonged *in vitro* cultivation of pre-implantation embryos, give advantages to transfer the embryos at the morula stage to uterine cavity (Chason *et al*., 2011). At this stage of natural development *in vivo*, the embryo enters the uterine cavity and interacts with its microenvironment. In addition, the transcription of the embryonic phenotype begins at the 3rd day of development and is actively progressing to day 4 (Kanka *et al.*, 2003). The development potential of embryos at stage of 2-8 blastomeres is difficult to be estimated by visual observation. It is shown that the prognostic signs of the implantation and pregnancy onset after the transfer of early embryos to the uterine cavity can be correlated with their morphological characteristics

and kinetics of development, which in turn often correspond with their chromosomal status (Magli *et al*., 2007). Despite the fact that embryos at the morula stage have been transferred since 1994 (Goto *et al.,* 1994; Huisman *et al*., 1994), an objective morphological evaluation for the day 4 embryos selection has not been developed, and those characteristics used for embryos of 1- 3 days can not be applied due to the complexity of blastomere number counting, estimating their size and morphology (Rienzi *et al*., 2005). The high outcome predictability after IVF using a combined score for zygote and embryo morphology and growth rate have been reported previously (De Placido *et al.,* 2002). The assess criteria tothe embryos at the stage of blastocysts (such as morphological evaluation of trophectoderm and intracellular cell mass) can be hardly used (Gardner & Schoolcraft, 1999).

It has been suggested to evaluate the morulae by the morphology and degree of compaction, as they correlated with the level of implantation and development potential rates (Ebner *et al*., 2009). A high degree of morula fragmentation reduces their implantation and, correspondingly, the pregnancy rate, while the presence of small amounts of fragmentation apparently has no adverse effect (Alikani *et al.,* 1999). Two distinctly different types of fragmentation were identified by Van Blerkom *et al.* in 2001, namely, the final fragmentation, representing stable, persistent fragments, clearly separated from blastomeres and the pseudo-fragmentation, which are characterized by a temporal appearance during or shortly after the cell division, but not found at later stages of development (Van Blerkom *et al*., 2001).

The compaction onset is an important prognostic criterion for the BFR and the potential for their implantation. Some authors state that the embryos with compaction at the third day of development have a high probability of their implantation (Le Cruguel *et al*., 2013). Early day 3 compaction may be a useful additional criterion for embryo transfer. Others believe that embryos that have

not reached the compaction stage up to day 4, but show mitotic activity to day 3, are comparable in BFR to those in which the compaction occurred on day 4. However, the decrease of BFR of embryos that remained at the same stage to days 3 and 4 has been noted (Fabozzi *et al.,* 2016). In addition, the implantation rate after transfer of day 4 compacted embryos is reported to be higher if compared with the embryos with early compaction (Tao *et al*., 2002; Feil *et al*., 2008).

Since the main trend in multiple pregnancy prevention and its' related risks is the transfer of one selected embryo, and with the aim of increasing the cumulative pregnancy rate, cryopreservation procedures should be used, for the proposal of prognostic criteria for evaluating the embryo cryoresistance are also important.

In this study, we evaluated the survival and BFR of day 4 embryos with different compaction degrees of blastomeres after cryopreservation. An osmotic reaction during exposure with cryoprotectant solutions was also evaluated. The blastomeres not included in the compaction were shown to be aneuploid (Lagalla *et al*., 2017). Moreover cryopreserved by slow cooling embryos at the stage morulae which had fragmentation more than 20%, had significantly lower post-thaw survival and transferable rates comparing with morulae which represented good quality and full compaction (Tao *et al*., 2004). Cryopreservation factors can lead to additional lesions of uncompacted blastomeres: increased osmotic pressure on the membrane cells during CPA exposure and wash out; toxic effects of CPAs; ice re-crystallization at the thawing stage (Men *et al*., 2005; Mazur & Paredes, 2016). Even more the toxic effect of high concentrations of CPAs used for embryo vitrification can lead to release of necrotic factors from weak non compacted cells of morulae. The factors can adversely affect the compacted part of embryo reducing their survival rate and future development.

Microsurgical removal of degenerated blastomeres of embryos was first carried out by M. Alikani (Alikani *et al*., 1993). This method was applied to

human embryos, cryopreserved using slow cooling rates (Rienzi *et al*., 2005 b; Elliott *et al.*, 2007; Liu *et al*., 2007). The removal of morulae non compacted cells and fragmentations is similar to blastomere biopsy for PGD which does not affect on the survival rate of embryo after vitrification-warming (Zhang *et al*., 2009).

Removal of cytoplasmic fragments in fresh embryo leads to an improvement of their quality and developmental ability (Alikani *et al*., 1993). This correlates with our results, which showed that the prior cryopreservation removal of fragments not included in compaction of day 4 embryo increased their survival rate and BFR, if the embryo compaction was 50% or higher (grade 2 and 3). Thus, this procedure allows to improve outcomes of freezing the morulae with an incomplete compaction grade 2 and 2, and thereby makes the higher chances of pregnancy occurrence. At the same time, the estimation of the proposed morula quality enables the prediction the survival rate of embryos after freeze-thawing and expediency of carrying out the biopsy of non-compacted areas. These data should be taken into account when counseling patients undergoing fertility treatment using assisted reproductive technology and cryopreservation methods.

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Ethical Standards

All the manipulations of gametes and embryos were performed according to the report of the Steering Committee On Bioethics (CDBI) on ‘The Protection of the human embryo in vitro’ CDBI-CO-GT3 (Strasbourg, 19 June 2003) with an informed patient consent and the decision of the Committee in Bioethics of the Institute for Problems of Cryobiology and Cryomedicine of the NAS of Ukraine.

Conflicts of interest

The authors have no conflict of interest with regard to these results.

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FIGURE LEGENDS

Figure 1 Grade of blastomere compaction degree. 1 – full compaction; 2–4 – a compaction occupies 75, 50, 25% of embryo volume, respectively; 5 – non-compacted morula.

Figure 2 Excluded fragments removal procedure. Embryo development to blastocyst stage, magnification x600, (n=55).

Figure 3 Survival rate of morulae with different grade of compaction after vitrification. A statistically significant difference was observed between vitrified morulae and biopsied morulae with the same grade of compaction marked with an asterisk (*) and between vitrified grade 1 morulae and the study group – (#), ($P<0.05$).

Figure 4 Blastocyst formation rate of morulae with different grade of compaction after vitrification. A statistically significant difference was observed between fresh morulae and vitrified morulae or biopsied morulae with the same grade of compaction marked with an asterisk (*) and between grade 1 fresh morulae and the study group – (#), ($P<0.05$).

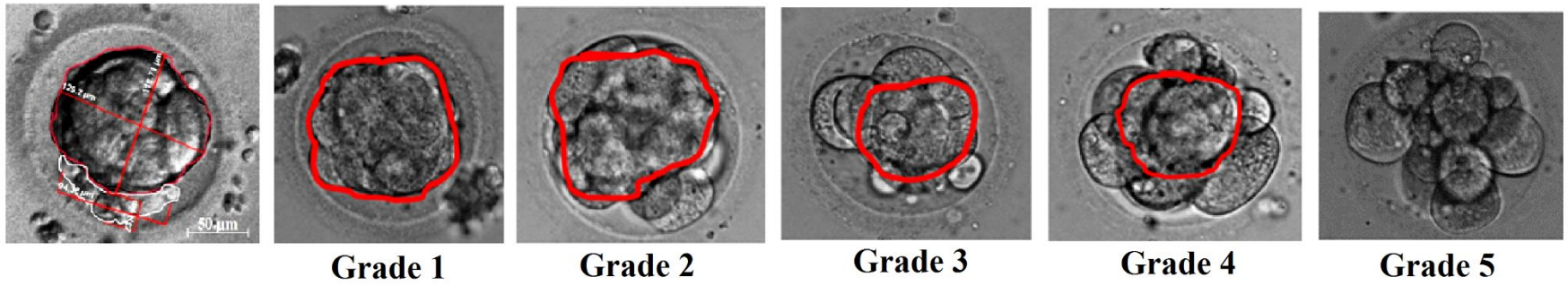

Figure 1.

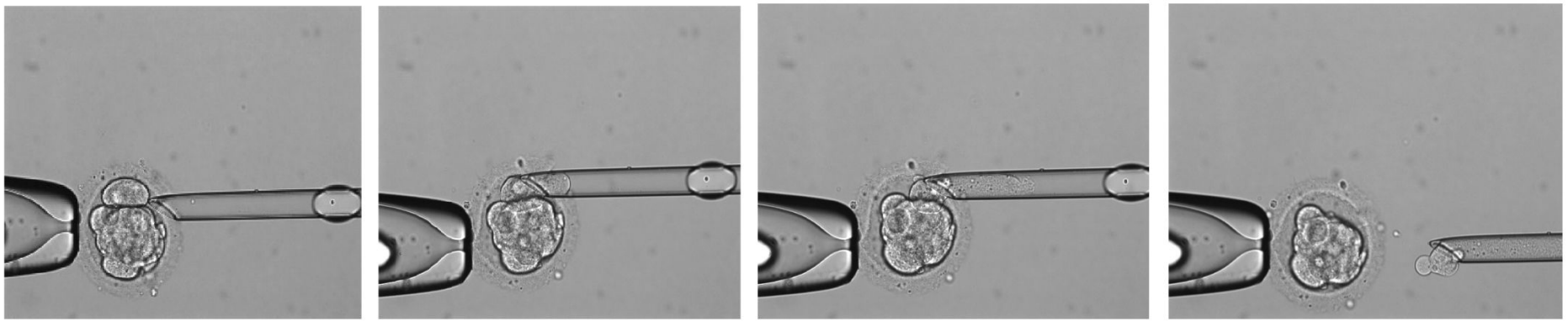

Figure 2

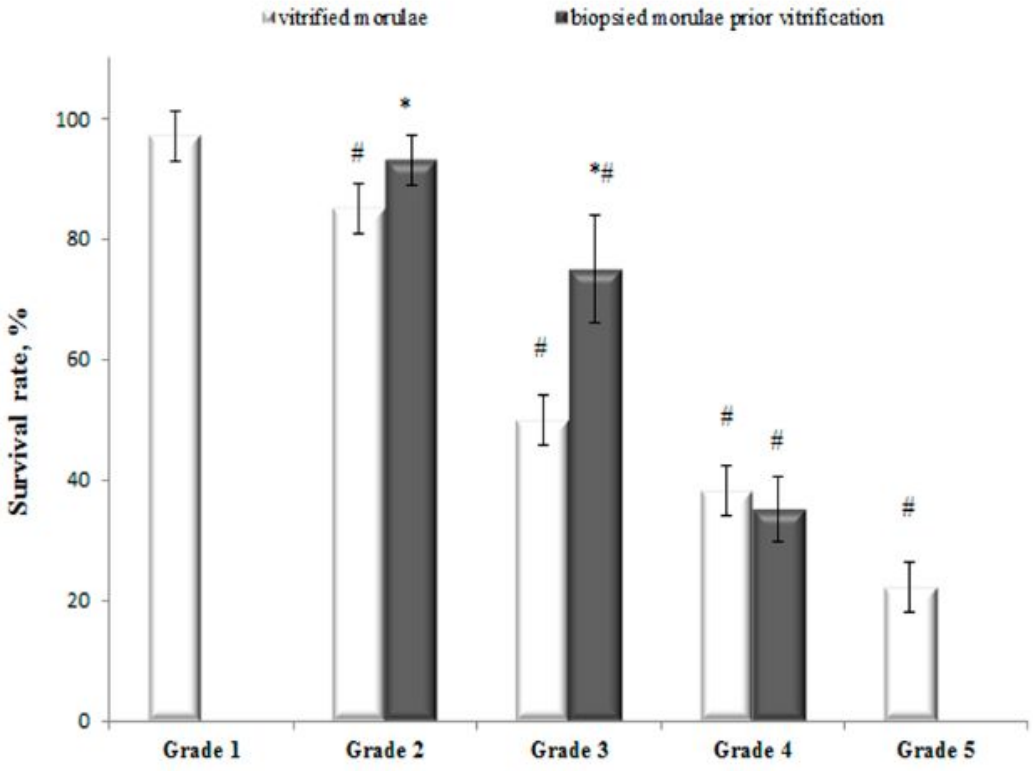
vitrified morulae
biopsied morulae prior vitrification
Survival rate, %
100
80
60
40
20
0
Grade 1
Grade 2
Grade 3
Grade 4
Grade 5
*
#
*#
#
#
#
#

Figure 3.

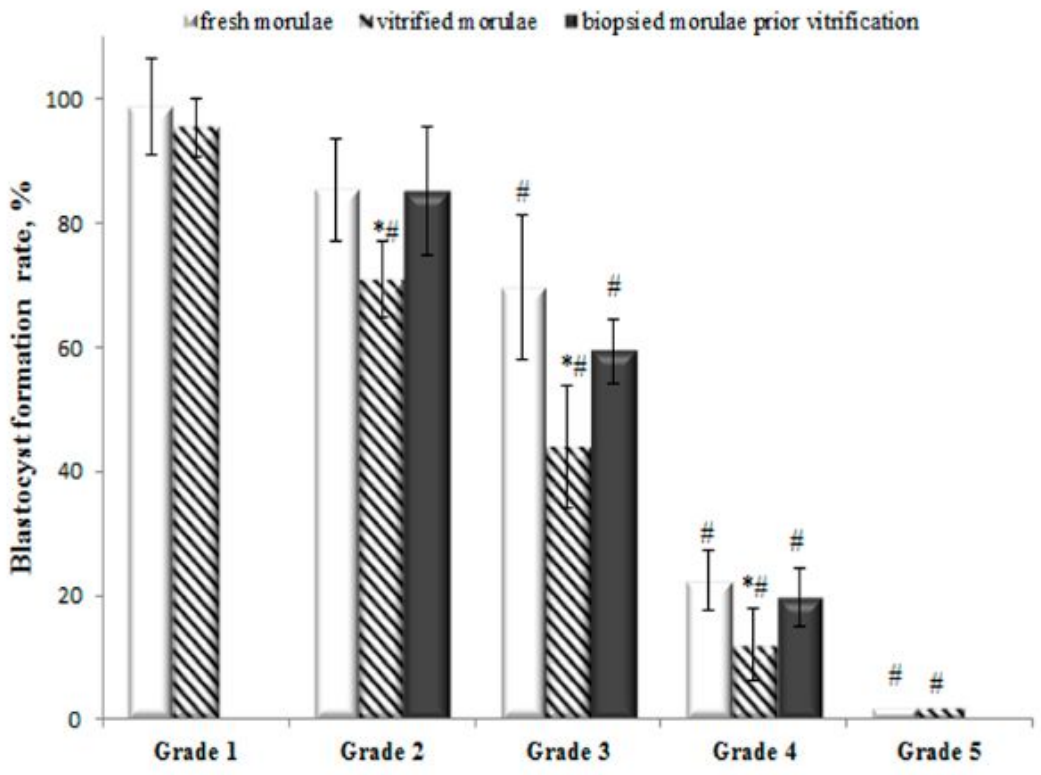

Figure 4.