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Intracytoplasmic Sperm Injection after Vitrification of Immature Oocytes in Follicular Fluid Increases Bovine Embryo Production

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ABSTRACT

***Background*:** Despite the low efficiency caused by its harmful effects, vitrification is the technique of choice for oocyte cryopeservation, especially at the germinal vesicle (GV) stage. This enables the banking of female gametes without linkage to the male genotype. Follicular fluid (FF), *in vivo*, is known to provide an adequate environment to the immature oocyte. The intra-cytoplasmic sperm injection (ICSI), by the other hand, can be used to bypass any sperm penetration disorder, including the ones caused by cryopreservation. This study aimed to evaluate oocyte vitrification in FF based solution, and to asses ICSI efficiency in the fertilization of vitrified/warmed bovine GV oocytes.

***Material, Methods & Results*:** Follicles of 2-8 mm in diameter were aspirated from bovine ovaries obtained from a slaughterhouse, selected and maintained into FF from aspiration, until their allocation in the experimental groups. The FF used to prepare the vitrification solution was centrifuged, heat inactivated, filtered through a 0.22 mm pore and stored at -20°C. Oocyte vitrification was done into one of these three solutions: The standard solution TCM-Hepes (TH-Vitri) was compared to a totally FF based solution (FF-Vitri), and to a 50:50 (v/v) mix of both solutions (TH:FF-Vitri). Oocytes were submitted to *in vitro* embryo production in order to assess embryo production efficiency. A second set of experiments using the FF-Vitri solution compared IVF versus ICSI. With basis on cleaved structures, the morula + blastocyst rate obtained in the Fresh Control (43.9%) was similar to FF-Vitri (31.1%). Conversely, the TH-Vitri (15.7%) and the TH:FF-Vitri (20.4%) rates were significantly lower than the Fresh Control. ICSI showed a positive effect in comparison with IVF. The embryo development rate of Vitri-IVF (18.8%) was the lowest, whereas Vitri-ICSI (37.3%) was similar to the Fresh-IVF (43.9%), but lower than the Fresh-ICSI (57.8%).

***Discussion*:** Oocytes cryopreserved in TH based solution are known to show certain rigidity in the zona pellucida, being this event a possible cause to spermatozoa penetration disruption. Our results agree with that, since the fertilization rate for TH-Vitri was significantly lower than for the FF-Vitri. In contrast, GV oocytes vitrified in total versus partial FF based solution showed similar maturation and fertilization rates as the Fresh Control, evidencing the beneficial effect of FF during the course of vitrification. It is possible that FF helped to adjust oocyte maturation, allowing a better nuclear-cytoplasmic synchrony. Also, it might have provided some protection due to its antioxidant properties. The releasing of cortical granules induced by freezing, lead to a zona pellucida hardening and failure in sperm penetration. Factors present in the FF might block this premature releasing of cortical granules, thus ensuring that the egg retains its ability to be fertilized after maturation. The blastocysts produced from the FF-Vitri oocytes were the only ones that had the average ICM similar to the Fresh Control, evidencing that besides the similarity in morula + blastocyst rates, the embryos derived from oocytes vitrified in FF solution have also yielded best quality. When vitrified warmed oocytes were submitted to ICSI, there was an increase in the blastocyst production. This increment of embryo production with ICSI evidences a pathway to overcome the zona pellucida biological barrier. In conclusion, the use of FF as base for vitrification solution improves further embryo development; ICSI increases the embryo production of vitrified/warmed bovine GV stage oocytes.

Keywords: oocyte cryopreservation, GV oocytes, ICSI, IVF, IVP, ART.

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INTRODUCTION

Vitrification is the technique of choice for cryopreserving oocytes, but further embryo development remain still poor, as a low percentage of oocytes remains competent to undergo normal development [11]. Results are still inconsistent due to harmful effects to the nucleus [13], to the zona pellucida [5], or to injuries in the cytoplasm.

Follicular fluid (FF), who has the ability to inhibit germinal vesicle breakdown of bovine oocytes [1] had some defined components already described, which are known to play important roles until the completion of fertilization. Such roles include inhibiting zona pellucida modifications [9,10], enhancing cytoplasmic maturation, and providing important promoting factors [21]. For the reasons stated here, FF makes a promising substance to be used in the course of GV stage oocyte vitrification.

The intra-cytoplasmatic sperm injection (ICSI), by the other hand, appears as a suitable tool to overcome the negative effects of oocyte cryopreservation. Indeed, previous reports showed ICSI to be successfully employed to increase embryo production rates of oocytes presenting an impaired quality [15,18].

This study aimed to evaluate the developmental efficiency and quality of embryos derived from GV oocytes vitrified in a base solution partially or totally composed of FF; and to compare the embryo production and quality after fertilizing oocytes using conventional *in vitro* fertilization (IVF) or ICSI, after their vitrification at the GV stage in FF based solution.

MATERIALS AND METHODS

All chemicals were purchased from Sigma-Aldrich[1].

Oocyte collection

Bovine ovaries obtained from a slaughterhouse had their follicles measuring 2-8 mm of diameter aspirated. Recovered cumulus-oocyte complexes (COCs) were maintained into FF throughout classification, being used only the ones classified as quality grade I or II [4]. Oocytes were maintained in FF (within up to 6 h after slaughter), until they were randomly allocated to the experimental groups.

Preparation of FF and vitrification base solutions

The FF used for vitrification was obtained as described for the oocyte collection, centrifuged at 5000 x *g* for 20 min, inactivated by heat at 45°C during 35 min, and filtered through a 0.22 mm pore, then stored at -20°C, until its use.

The three different vitrification solutions were: standard Hepes-buffered TCM-199 + 20% of estrous mare serum (TH-Vitri) solution, total FF base solution (FF-Vitri), and 50:50% (v/v) TH and FF base (TH:FF-Vitri) solution.

Before mixing with cryoprotectants, each solution was stabilized at 7°C for 30 min to avoid precipitation of FF proteins during mixing.

Experimental design

The first experiment (four replications) compared GV oocyte vitrification in a base solution totally (FF), partially (TH:FF), or not composed (TH = standard solution) of follicular fluid. The FF based solution showed the best result, so the second experiment (five replications) compared IVF versus ICSI to fertilize GV stage oocytes previously vitrified in FF based solution (fresh oocytes were also fertilized to be used as controls). Viability criteria were based on: maturation and fertilization rates at D1 (first experiment); and in the rates of cleavage at D2, morula and blastocyst at D7, and in the blastocyst quality (first and the second experiments).

Oocyte vitrification and warming

Oocytes to be vitrified in TH based solutions (TH or TH:FF) were exposed to them for 8 to 10 min prior to the onset of vitrification steps, being the mix with cryoprotectants performed on a cooled (7°C) surface. The vitrification dish was then warmed at 38.5°C for 30 min. The vitrification steps consisted in the exposure of 3 to 5 oocytes to the base solution containing 10% of dimethylsulfoxide (DMSO) and 10% of ethylene glycol (EG), for 30 s and then to 20% DMSO and 20% EG. Oocytes were aspirated (by capillarity) to the tip-end of a glass pipette, and immediately plunged into liquid nitrogen in the time lapse of 20 to 25 s. The vitrified oocytes were maintained for at least 1 h into the liquid nitrogen (LN_2), before proceeding to the 2-step warming. To that, the glass pipette was removed from the LN^2 and immersed into TH solution added of 0.4 M of sucrose (first step) at 38.5°C, for 5 min, being the released oocytes transferred to TH added of 0.2 M sucrose (second step) for additional 5 min. After warming, oocytes were maintained into TH solution for 10 min, and finally transferred to the *in*

vitro maturation (IVM) dish. A group of oocytes was maintained as a Fresh Control.

IVM procedure and incubation conditions

Oocytes were maturated in groups of 30-40, into 4-well dishes containing 400 µL of modified tissue culture medium, added of 5.95 mg/mL of HEPES, 2.62 mg/mL of $NaHCO_3$, 0.025 mg/mL of sodium pyruvate, 0.01 IU/mL of FSH, 0.5 µg/mL of LH and 10% of estrus mare serum (EMS). The maturation was performed at 38.5°C and 5% CO_2 in air and saturated humidity, for 20 to 24 h.

Semen preparation and in vitro *fertilization (IVF)*

A sample of frozen-thawed semen from a *Bos taurus* bull was thawed in water bath at 37°C for 20 s and selected by mini-Percoll [12] gradients of 90 and 45%. Selected sperm cells were adjusted to a concentration of 1.5 x 10^6 spermatozoa/mL. Then they were co-incubated with the oocytes during 18-20 h, into 4-well dishes containing 400 µL of Talp-Fert [16] medium added of 6 mg/mL of bovine serum albumin (BSA), 0.022 mg/mL of sodium pyruvate, 30 µg/mL of heparin and PHE (penicillamine, hypotaurine and epinephrine). The incubation conditions were the same as described for IVM.

Assessment of nuclear status of presumptive zygotes

After IVF, cumulus cells were removed from presumptive zygotes by successive pipetting in TH, being then fixed in absolute ethanol with 10 µg/mL bisbenzimide (Hoechst 33342) and placed in a glycerol drop between glass slide and coverslip. The evaluation was performed through epi-fluorescent microscope[2] to determine the nuclear developmental status, as previously described [20], being classified as: 1- maturated oocytes; 2- penetrated oocytes. Penetrated oocytes were subclassified in either: 2.1- polyspermic zygotes, or: 2.2- fragmented zygotes.

Intracytoplasmic sperm injection (ICSI)

ICSI and oocyte activation procedures were performed as previously described [16], using a micromanipulation station (Narishige IM-9B)[3] set up with an inverted microscope (Olympus IX51)[2]. After IVM oocytes had their cumulus cells removed by successive pipetting in TH, selected upon the presence of the first polar body and placed into 25 µL droplets of Hepes-buffered TCM-199 added of 6 mg/mL of BSA, under mineral oil, in groups of 5 MII oocytes.

Previously selected (by mini-Percoll) spermatozoa were maintained into a droplet (15 µL) of PVP-40 (10% polyvinylpyrrolidone, in HEPES-buffered TCM-199). A motile sperm was aspirated into the injection micropipette; the ooplasm of the oocyte (attached to a holding micropipette) was strongly aspirated until its oolema was broken down to allow the sperm to be injected into the oocyte cytoplasm. Oocytes were activated by 5 min exposure to 5 µM of ionomycin and 2.5 - 3 h incubation in 2 mM of 6-Dimethyl aminopurine. The incubation conditions were the same as described for IVM and IVF. Activated oocytes were then placed in the *in vitro* culture (IVC) dish.

In vitro *culture (IVC)*

Embryos were cultured into 4-well dishes containing 400 µL of SOFaaci medium [8] added of 5% EMS under mineral oil, as previously described [15]. The first 2 days of culture were performed at 38.5°C with 5% CO_2 in air and saturated humidity. From day two to day seven, the IVC dish was sealed into a foil bag, filled with lung air, blown (always from the same operator) after 4 s of apnea. The lung air provided a 5% CO_2 atmosphere (data not shown), and was replaced on day five.

Total Cell Number (TCN) and Inner Cell Mass (ICM) proportion

A previously described differential staining technique [14] was used to estimate the total cell number (TCN) and the proportion of inner cell mass (ICM) of the blastocysts.

Day seven blastocysts were incubated in 10 mg/mL of propidium iodide and 1 mg/mL of Triton X-100, diluted in Dulbecco's phosphate-buffered saline (D-PBS), for 40 s at room temperature, being immediately fixed with absolute ethanol added of 15 mg/mL of bisbenzimide (Hoecsht 33342) for additional 7 min. Fixed embryos were placed into a 5 µL droplet of glycerol, between slide and coverslip, and evaluated through epi-fluorescent inverted microscope[2]. The TCN and the number of cells in the trophectoderm were assessed, being the ICM proportion determined by the difference between these two estimates.

Statistical Analysis

Data were analyzed using the JMP software version 5, 2002 (SAS)[4]. Cleavage and blastocyst rates from both experiments were analyzed through the Chi-

Square test. The rates of oocyte maturation, penetration and fragmentation, as well as blastocyst TCN and proportion of ICM were analyzed by ANOVA (normalized by the arc sin of the square root of the data), and Tukey test. The significance level was always of 5%.

RESULTS

Maturation and fertilization patterns after immature oocyte vitrification in a base solution partially of totally composed of FF

The maturation rate of oocytes vitrified in TH (66.7%) was significantly lower than the Fresh Control (86.8%). However, when oocytes were vitrified in TH:FF (75.8%) or in FF (78.0%), they no longer showed statistical differences with other groups, nor between them (Table 1). Fertilization rates were similar for both Fresh Control (94.3%) and FF-Vitri (92.7%), both significantly higher than TH-Vitri (75.6%), being the TH:FF-Vitri (83.5%) similar to all other treatments. All the vitrified groups showed more polyspermic and fragmented zygotes ($P \leq 0.05$) than the Fresh Control (Table 1).

Assessment of embryo development rates and quality after immature oocyte vitrification in a base solution partially of totally composed of FF

The experimental groups that had FF in its base solution showed lower ($P \leq 0.05$) cleavage rates (TH:FF 51.9% and FF 45.9%) than the Fresh Control (66.0%), but the cleavage rate from TH-Vitri (55.4%) was similar to all other groups (Table 2). Blastocyst and morula + blastocyst rates at day 7 were lower in all vitrified groups, if compared to the Fresh Control ($P \leq 0.05$). Conversely, if estimated based on cleaved, morula + blastocyst rates were similar ($P > 0.05$) for the Fresh Control (43.9%) and FF-Vitri (31.1%). The average TCN of blastocysts did not differ ($P > 0.05$) among the groups Fresh Control (59.8), TH-Vitri (54.6), TH:FF-Vitri (59.7), and FF-Vitri (67.2). Despite being a cryopreserved group, the blastocysts from FF-Vitri (20.5%), had similar ($P > 0.05$) ICM proportion (Table 2) as the Fresh Control (24.9%), in contrast with the lower proportion of their other vitrified counterparts (TH-Vitri 18.1% and TH:FF-Vitri 18.0%).

Table 1. Evaluation of nuclear status of IVF zygotes derived from fresh or vitrified immature bovine oocytes.

Group	Maturated oocytes n (%)	Fertilyzed oocytes n (%)	Polyspermic zygotes n (%)	Fragmented zygotes n (%)
Fresh Control	105/121 (86.8)^{a}	99/105 (94.3)^{a}	0/99 (0.0)^{b}	0/99 (0.0)^{b}
TH-Vitri	86/129 (66.7)^{b}	65/86 (75.6)^{b}	7/65 (10.8)^{a}	6/65 (9.2)^{a}
TH:FFVitri	91/120 (75.8)^{ab}	76/91 (83.5)^{ab}	7/76 (9.2)^{a}	7/76 (9.2)^{a}
FF-Vitri	96/123 (78.0)^{ab}	89/96 (92.7)^{a}	7/89 (7.9)^{a}	7/89 (7.9)^{a}

^{ab}Distinct letters in the same column shown difference ($P \leq 0.05$). TH-Vitri - Vitrified oocytes in TCM-199 HEPES + 20% estrus mare serum as base solution. TH:FF-Vitri - Vitrified oocytes in mix (50/50%) of TCM-199 HEPES + 20% estrus mare serum and follicular fluid as base solution. FF-Vitri - Vitrified oocytes in follicular fluid as base solution.

Table 2. Evaluation of embryo development rates, average total cell number and inner cell mass (ICM) ± SE proportion of IVF blastocysts derived from fresh or vitrified immature bovine oocytes.

Group	Cultured n	Cleaved n (%)	Blastocyst n (%)	Morula + blastocyst		Cells in blastocysts	
				From culture n (%)	From cleaved (%)	Total cell number	Cells in ICM (%)
Fresh Control	100	66(66.0)^{a}	23(23.0)^{a}	29(29.0)^{a}	43.9^{a}	59.8 ± 4.7	24.9 ± 1.9^{a}
TH-Vitri	92	51(55.4)^{ab}	5(5.4)^{b}	8(8.7)^{b}	15.7^{b}	54.6 ± 5.3	18.1 ± 2.1^{b}
TH:FFVitri	104	54(51.9)^{b}	4(3.8)^{b}	11(10.6)^{b}	20.4^{b}	59.7 ± 2.4	18.0 ± 1.0^{b}
FF-Vitri	98	45(45.9)^{b}	4(4.1)^{b}	14(14.3)^{b}	31.1^{ab}	67.2 ± 5.3	20.5 ± 2.1^{ab}

^{ab}Distinct letters in the same column shown difference ($P \leq 0.05$). TH-Vitri - Vitrified oocytes in TCM-199 HEPES + 20% estrus mare serum as base solution. TH:FF-Vitri - Vitrified oocytes in mix (50/50%) of TCM-199 HEPES + 20% estrus mare serum and follicular fluid as base solution. FF-Vitri - Vitrified oocytes in follicular fluid as base solution.

Embryo development rates and quality after IVF or ICSI of vitrified/rewarmed immature oocytes

The cleavage rate for Fresh-IVF (65.6%) was higher ($P \leq 0.05$) than for all the other groups (Fresh-ICSI 50.0%, Vitri-IVF 48.3% and Vitri-ICSI 46.9%) [Table 3]. Blastocyst rates were higher ($P \leq 0.05$) for fresh-IVF (26.4%) and fresh–ICSI (28.9%) than for Vitri-IVF (4.9%) and Vitri-ICSI (11.9%), being Vitri-ICSI also higher than Vitri-IVF (Table 3). When morula + blastocyst rates were calculated with basis on cleaved, this rate was similar between Fresh-IVF (43.9%) and Fresh-ICSI (57.8%), being Fresh-IVF and Vitri-ICSI (37.3%) similar between them, but lower than Fresh-ICSI. The lowest rate ($P \leq 0.05$) in comparison to all other groups (Table 3) was presented by Vitri-IVF (18.8%).

The mean TCN, as well as the ICM proportion of blastocysts from all groups did not differ ($P > 0.05$) amongst them, being the TCN respectively 58.5, 69.0, 81.7 and 73.5 for Fresh-IVF, Fresh-ICSI, Vitri-IVF and Vitri-ICSI. The ICM proportion was respectively of 22.3, 21.0, 26.9 and 21.6 for Fresh-IVF, Fresh-ICSI, Vitri-IVF and Vitri-ICSI.

Table 3. Evaluation of embryo development rates, average total cell number and inner cell mass (ICM) ± SE proportion of IVF or ICSI blastocysts derived from fresh or vitrified immature bovine oocytes.

Group	Cultured n	Cleaved n (%)	Blastocyt n (%)	Morula + blastocyst		Cells in blastocysts	
				From cultured n (%)	From cleaved (%)	Total cell number	cells in ICM (%)
Fresh-IVF	125	82(65.6)[a]	33(26.4)[a]	36(28.8)[a]	43.9[ab]	58.5 ± 5.4	22.3 ± 1.2
Fresh-ICSI	128	64(50.0)[b]	37(28.9)[a]	37(28.9)[a]	57.8[a]	69.0 ± 4.8	21.0 ± 1.1
Vitri-IVF	143	69(48.3)[b]	7(4.9)[c]	13(9.1)[c]	18.8[c]	81.7 ± 11.3	26.9 ± 2.5
Vitri-ICSI	160	75(46.9)[b]	19(11.9)[b]	28(17.5)[b]	37.3[b]	73.5 ± 6.8	21.6 ± 1.5

[abc]Distinct letters in the same column shown difference ($P \leq 0.05$).

DISCUSSION

Despite the damage caused by cryopreservation, the maturation and fertilization rates after vitrification of GV oocytes in total or partial FF based solution were similar to the Fresh Control (Table 1), evidencing its beneficial effect in the course of vitrification. It is possible that substances present in the FF give them some protection during cryopreservation. This corroborates with previous findings [1], that small follicles have best ability to inhibit the germinal vesicle breakdown.

Reports that oocytes cryopreserved in TH show rigidity in the zona pellucida [20] are a possible link to an impaired spermatozoa penetration [2,3,5]. Our findings agree with that, since the fertilization rate of TH-Vitri was lower than the FF-Vitri (Table 1). Oocytes vitrified in total FF solution yielded a fertilization rate similar to the Fresh Control. The early releasing of cortical granules induced by freezing and thawing is another possible reason to failure in sperm penetration [7]. Indeed, it should be investigated whether FF is responsible for triggering some pathway to prevent this early release of cortical granules, when applied during the course of vitrification.

Intriguingly, the cleavage rates for both treatments that vitrified using FF had the cleavage rate lower than the Fresh Control, unlike the TH-Vitri (Table 2). This needs further investigation in order to pinpoint the reasons why this pattern was observed in the cleavage rates. Still regarding the vitrified/rewarmed oocytes, the blastocysts from the FF-Vitri were the only ones showing the ICM percentage similar to the Fresh Control (Table 2), evidencing that embryos derived from oocytes vitrified in FF had an improved quality. Previous studies have reported that FF contains antioxidants such as catalase [6], which may play a positive role in the defense of oocytes during vitrification.

When vitrified-warmed oocytes were fertilized by ICSI, the blastocyst production was increased (Table 3). This increased embryo production, as previously found in humans [18], evidences that ICSI is an alternative to overcome the zona pellucida barrier, as the sperm cell is inserted directly into the ooplasm. Our data also show increased results of Vitri-ICSI. Conversely, previous findings in the bovine [17] did

not find any increment in embryo development after MII oocyte vitrification and ICSI, though under their conditions the cleavage rates increased. This study shows that: the composition of the base solution for oocyte vitrification can improve the embryo production outcome, and ICSI improves the embryo production for previously vitrified oocytes. The roles played by the FF during the course of oocyte vitrification should be further investigated. Ultimately, the post-implantation efficiency of ICSI in post-vitrified oocytes should be evaluated, after embryo transfer.

CONCLUSIONS

Vitrification of GV stage oocytes in FF based solution has a positive effect in the maturation, fertilization and embryo development. Vitrification of GV oocytes in a solution totally based in FF improves further embryo quality. In the comparison between ICSI and IVF for the fertilization of oocytes vitrified in FF, ICSI provides the best embryo development rates.

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***Declaration of interest*.** The authors declare that they have no conflict of interest that is directly or indirectly related to this research.

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