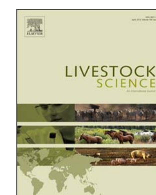
Livestock Science 197 (2017) 96–101

Contents lists available at ScienceDirect

Livestock Science

journal homepage: www.elsevier.com/locate/livsci



Short term culture with cAMP modulators before vitrification significantly improve actin integrity in bovine oocytes

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ARTICLE INFO

Keywords:
Forskolin
IBMX
Vitrification
Pre-IVM
VG oocytes

ABSTRACT

Oocyte cryopreservation is a strategic tool for assisted reproduction, but has limited use due to the complex cellular structure of oocytes, which leads to sub-optimal survival rates. In this study, we used the SPOM *in vitro* maturation system, which is based on supplementation of cAMP modulators in order to extend meiotic arrest and improve oocyte maturation. cAMP modulators (Forskolin and IBMX) were administered in a short term culture (STC) before or after vitrification, followed by an extended maturation with cilostamide. We hypothesized that a STC with cAMP modulators would improve immature oocyte health and enhance cryotolerance. We found vitrification caused oocyte damage in a great extent, impairing nuclear maturation rates in all vitrified groups (Percentage of matured oocytes: CONT FRESH 77.8[c]; CONT VIT 31.4[ab]; STC/VIT 39.5[b]; VIT/STC 18.6[a]). Vitrification also promoted degradation of cytoskeletal actin filaments (Percentage of Injured oocytes: CONT FRESH 0.0[a]; CONT VIT 50.0[b]; STC/VIT 39.7[b]; VIT/STC 74.0[c]), and increased calcium release (Calcein-AM mean ± SD: IMMATURE 1.0 ± 0.49; VIT 1.76 ± 1.13; STC 1.38 ± 0.95; STC/VIT 1.58 ± 0.99). However, STC seemed to attenuate negative effects of vitrification, since oocytes subjected to STC prior to vitrification presented predominance of polymerized actin filaments (Percentage of Intact oocytes). Unfortunately, embryo cleavage rate (SPOM 73.66[a]; STC/VIT 7.91[b]; VIT/STC 4.62[b]) and blastocyst development rate (SPOM 25.14[a]; STC/VIT 1.34[b]; VIT/STC 0.00[b]) was impaired in vitrified groups, regardless STC treatment. In conclusion, STC with cAMP modulators, Forskolin and IBMX, decreases cytoskeleton actin filaments injuries caused by oocyte vitrification, which may consequently increase oocyte viability. Our results suggest STC should be considered and improved for immature oocyte vitrification systems.

1. Introduction

Oocyte cryopreservation is considered a strategic tool for embryo *in vitro* production (IVP) in livestock, facilitating transport and commercialization of these gametes. It is also a technic often applied before cancer treatment, in order to preserve the patient's capability of conceiving (Mahajan, 2015). In addition, genetic conservation and preservation of endangered-species can also be achieved by oocyte cryopreservation (Andrabi and Maxwell, 2007). However, reduced survival of frozen oocytes results in low rates of embryo development and pregnancy (Vajta et al., 1998; Lane and Gardner, 2001), limiting its use in routine.

Compared to blastocysts, oocytes are particularly challenging to cryopreserve due to their large cell size (Chen et al., 2003) and plasma membrane singularities (Jin et al., 2011). Also, oocytes have a higher lipid content compared to blastocysts (Ruffing et al., 1993). Those characteristics hinder the penetration of cryoprotectants, increasing oocyte sensitivity to cryopreservation process.

As a result of the reduced cryotolerance, oocytes display morphological and physiological damages after cryopreservation, mostly involved with cytoplasmic membrane, cytoskeleton and intercellular junctions (Vajta, 2000). In addition, hardening of the zona pellucida, due to premature exocytosis of cortical granules, can impair fertilization (Mavrides and Morroll, 2005), resulting in considerable decrease

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http://dx.doi.org/10.1016/j.livsci.2017.01.013
Received 15 June 2016; Received in revised form 18 January 2017; Accepted 25 January 2017
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in embryonic development potential. In this context, improvement of oocyte quality may be a valuable strategy to enhance its viability after cryopreservation.

Recent studies demonstrated that short term culture (STC) with cyclic adenosine 3′,5′-monophosphate (cAMP) modulators, such as Forskolin (FK) and 3-Isobutyl-1-methylxanthine (IBMX), prior to *in vitro* maturation (IVM) promotes a significant improve in oocyte quality, resulting in benefits to embryo development (Rose et al., 2013; Richani et al., 2014; Appeltant et al., 2015; Ulloa et al., 2015). Those studies aimed to prolong the meiotic arrest in an attempt to offer to the oocytes *in vitro* conditions more similar to those found *in vivo*, thus producing structures with greater development competence.

cAMP is an important molecule in signal transduction within the cell, functioning as a second cell messenger of gonadotrophin stimulation. It is known that its levels are controlled through modulation of its synthesis by adenylyl cyclase (AC) enzyme at the oocyte (Kuyt et al., 1988) and the granulosa cells (Thomas et al., 2004), and by degradation, through the cyclic nucleotide phosphodiesterase (PDE) enzymes within the cumulus-oocyte complexes (COCs) (Sirard and Bilodeau, 1990). High levels of cAMP keep the meiosis block suppressing activation of maturation promoting factor (MPF), and low levels leads to MPF activation, resulting in germinal vesicle breakdown and resumption of meiosis (Mermillod et al., 2000; Bilodeau-Goeseels, 2011).

In addition, STC with cAMP modulators increases the extent of communication between the oocyte and cumulus cells by maintaining the integrity of gap junctions (Albuz et al., 2010). Communication between oocyte and cumulus cells provides essential metabolic support for the oocyte growth, maturation and embryo development after fertilization (Li and Albertini, 2013), besides it is related with the regulation of anti-oxidation and redox potential (Combelles et al., 2009; Li et al., 2011).

The STC with cAMP presents encouraging results regarding oocyte quality, therefore we believe this system could be beneficial to vitrified oocytes. In the present study, we analyzed viability parameters such as nuclear maturation, cytoskeleton integrity and gap junctional activity, since the disruption of those are often side effects of vitrification procedure, besides cleavage and embryo development. Therefore, the aim of this study was to use the STC with cAMP modulators in order to enhance oocyte cryotolerance.

2. Material methods

2.1. Reagents

All chemicals used in this study were purchased from Sigma-Aldrich Chemical Co. unless otherwise stated.

2.2. Experimental design

The main purpose of this study was to evaluate the effect of STC with cAMP modulators before and after vitrification/warming (VIT) on oocyte viability parameters (i) nuclear maturation, (ii) actin filaments integrity, (iii) gap junctions activity, besides embryo *in vitro* production. For that, experiments were designed in two assays.

In assay 1 we tested the effect of the *simulated physiological oocyte maturation* (SPOM), a two-step IVM system comprising of (i) a STC with cAMP modulators (originally named as pre-IVM) +(ii) extended IVM (Albuz et al., 2010). Step (i) STC was employed before (STC/VIT group) and after (VIT/STC group) vitrification of immature oocytes. After maturation period, oocytes were fixed for nuclear maturation and actin filaments analyses. For controls of those analysis immature oocytes were VIT (CONT VIT group) our not (CONT FRESH group), than subjected to IVM in standard TCM based medium. Gap juntions activity was assessed right after VIT (VIT group), STC followed by vitrification/warming (STC/VIT group) and STC alone (STC group). Immature oocytes were controls for that analysis.

In addition, assay 2 tested *in vitro* embryo production within groups: fresh oocytes matured in SPOM system (SPOM group), STC/VIT group and VIT/STC group.

2.3. Oocyte collection and selection

Ovaries were collected at local abattoir and processes within two hours after killing. The ovaries were washed in saline at 35–37ºC and follicles measuring 3–8 mm in diameter were aspired using an 18 G needle coupled to a 20 ml syringe. COCs having at least three cumulus cells layers and homogeneous cytoplasm were selected under a stereomicroscope and washed in TCM-199 buffered with HEPES (Gibco BRL, Grand Island, NY) supplemented with 1.0 mM sodium pyruvate (Gibco BRL, Grand Island, NY) and 100 UI penicillin and 0.1 mg/ml streptomycin.

2.4. Oocyte vitrification and warming

Vitrification media (BM: base media; VT1: vitrification solution one, VT2: vitrification solution two) was prepared according to (Vajta et al., 1998), with minor modifications. First, oocytes were washed in BM (TCM-199 buffered with HEPES (Gibco BRL, Grand Island, NY) supplemented with 1.0 mM sodium pyruvate (Gibco BRL, Grand Island, NY), 100UI penicillin, 0.1 mg/ml streptomycin, and 20% fetal calf serum (FCS)). Then they went through two step vitrification media (VT1, VT2) containing increasing concentrations of cryoprotectant, dimetilsulfoxide (DMSO) and ethylene glycol (EG). VT1 consisted in BM with 7.5% DMSO and 7.5% EG, wherein the oocytes remained for three minutes. VT2 consisted in BM with 16.5% DMSO and 16.5% EG, in which each oocyte remained 30–40 s before being deposited on the vitrification device (WTA®, Watanabe Applied Technology Ltd, Brazil) and dipped into liquid nitrogen.

For warming, oocytes were plunged in BM with 0.5 M Sucrose solution for five minutes, and then were washed for one minute in BM, to remove the sucrose.

2.5. IVM

In VIT group IVM was performed in TCM-199 supplemented with 0.2 mg/ml BSA, 1.0 mM sodium pyruvate (Gibco BRL, Grand Island, NY), 0.5 mg/ml FSH (Folltropin™, Bioniche Animal Health, Belleville, ON, Canada), 10 IU/ml of hCG, 1 µg/ml β-estradiol, 12.5 µg/ml recombinant human insulin, 6.875 µg/ml human transferrin (substantially iron-free), and 6.25 ng/ml sodium selenite (ITS) and 100 UI penicillin, 0,1 mg/ml streptomycin for 24 h at 38.5 °C, 5% CO2 in atmospheric air and maximum humidity.

In assays 1 and 2, the SPOM IVM protocol was adapted from Albuz et al. (2010). The step 1 of SPOM system (STC), was carried out in 100 µl /10–20 COCs in TCM-199 buffered with HEPES and supplemented with 0.4 mg/ml BSA, 1.0 mM sodium pyruvate (Gibco BRL, Grand Island, NY, USA), 12.5 µg/ml recombinant human insulin, 6.875 µg/ml human transferrin (substantially iron-free), and 6.25 ng/ml sodium selenite (ITS) and 100 UI penicillin, 0,1 mg/ml streptomycin, 100 µM FK and 500 µM IBMX, and kept for 2 h at 38.5 °C. Then SPOM- extended IVM was performed in 500 µl of IVM medium composed of TCM-199 supplemented with 0.2 mg/ml BSA, 1.0 mM sodium pyruvate (Gibco BRL, Grand Island, NY), 0.5 mg/ml FSH (Folltropin ™, Bioniche Animal Health, Belleville, ON, Canada), 10 IU/ml hCG, 1 µg/ml β-estradiol, 12.5 µg/ml recombinant human insulin, 6.875 µg/ml human transferrin (substantially iron-free), and 6.25 ng/ml sodium selenite (ITS) and 100 UI penicillin, 0,1 mg/ml streptomycin, with 20 µM cilostamide, and cultured for 28 h at 38.5 °C, 5% CO2 in atmospheric air and maximum humidity.

2.6. IVF / IVC

In assay 2, groups (SPOM, STC/VIT and VIT/STC) were subjected to IVF and IVC. Briefly, after SPOM IVM, groups of 10–20 oocytes were washed twice and transferred to 30 µl drops of commercial IVF medium (Bioklone® Reprodução Animal, São Paulo, Brazil) covered with sterile mineral oil. For IVF frozen straws of conventional semen of the same bull were used. Each straw containing 20 million spermatozoa was centrifuged on a discontinuous 45/90 Percoll gradient for 7 min at 3600*g*. The 80 µl pellet was ressuspended in 1.0 ml IVF medium and again centrifuged for 5 min at 520*g*. After centrifugation, 50 µl of the medium containing the pellet was collected from the bottom of the tube and homogenized in a conic tube. The final suspension was divided among ten drops containing one to ten oocytes or five drops containing ten to 20 oocytes, in a final concentration of 5×10^3 spermatozoa for each oocyte. The plates were incubated at 38.5 °C for 20 h in an atmosphere of 5% CO2 in air under saturated humidity. After IVF, presumptive zygotes were partially denuded of cumulus cells by vigorous pipetting and groups of 15–20 presumptive zygotes were cultured in 100 µl drops of commercial SOF medium (Bioklone® Reprodução Animal, São Paulo, Brazil) covered with sterile mineral oil at 38.5 °C in an atmosphere of 5% CO2 in air under saturated humidity for seven days. Cleavage rate was assessed 48–72 h after IVF and blastocyst rate at d7 of development.

2.7. Nuclear maturation

For analysis of nuclear maturation, oocytes were denuded in 2 mg/ml hyaluronidase solution, fixed in 4% paraformaldehyde (PFA) solution for 30–40 min, and stored at 4 °C. At the time of analysis oocytes were stained with Hoechst 33342 and evaluated in a fluorescence microscope (Olympus IX-70, Tokyo, Japan) at a wavelength between 340–380 nm, for nuclear configuration in germinal vesicle (GV), metaphase I (M I) and metaphase II (M II), to obtain the rate of matured (MII) oocytes in each experimental group. Immature oocytes (n=15) were stained side by side to guarantee the reliability of the assay, and displayed 93.3% GV oocytes.

2.8. Actin integrity

Cytoskeletal integrity analysis was performed by the evaluation of actin filaments. Denuded and PFA-fixed oocytes were stained with Phalloidin (Phalloidin Atto-532; 1:30 in concentration) for 30 min. Oocytes were evaluated under a fluorescence microscope (Olympus IX-70, Tokyo, Japan) with a wavelength between 568–592 nm. Images of each structure were recorded using AxioCam camera and 4.7.1 AxioVision software (Carl Zeiss, Jena, Germany). In our assay, Phalloidin staining pattern was read as intact, when oocyte was stained, or injured, when oocyte was not stained, as Phalloidin stain binds strongly to polymerized form of actin, F-actin. Immature oocytes (n=9) were stained before any treatment as a control of the staining. Those displayed 100.0% of the intact pattern (stained).

2.9. Gap junctional activity

Gap Junctional activity was estimated by transfer of substances between the cumulus cells and oocyte, using a calcein-AM staining protocol (Thomas et al., 2004). Calcein-AM passively diffuses into the cells and when the AM portion is cleaved releasing the calcium binding site of calcein, it fluoresces. The calcein, being negatively charged, does not leave the cell by plasma membrane, only by gap junctions.

To perform this evaluation, the oocytes were transferred to medium containing calcein AM (1 mM) in the absence of phenol red and proteins for 15 min. Then, structures were transferred to calcein AM free medium for 25 min, and washed three times. After, COCs were denuded and oocytes were evaluated in a fluorescence microscope (Olympus IX-70, Tokyo, Japan) in a 496–516 nm wavelength. Images of each structure were recorded using AxioCam camera and 4.7.1 AxioVision software (Carl Zeiss, Jena, Germany) and measured for fluorescent intensity by ImageJ software. The mean fluorescence intensity of each oocyte was recorded, and results were normalized by the mean values of IMMATURE group. The final data refers to relative florescence intensity, reflecting gap activity in comparison with immature untreated oocytes, considered the expected pattern for this analysis. A second analysis was performed wherein the structures have been divided according to the staining patterns observed (Low, Middle and High intensity).

2.10. Statistical analyses

Rates of matured oocytes (MII), cleavage and blastocyst were evaluated by chi-square test ($\chi2$). As for the gap junctions activity, the results were submitted to nonparametric Kruskal-Wallis test and Dunn post-test. The percentages of staining patterns and integrity of the cytoskeleton (actin) were compared between groups using Fisher's exact test. Analyses were performed in GraphPad INSTAT, at the significance level of 5%.

3. Results

3.1. Assay 1: effect of STC with cAMP modulators before and after vitrification on oocyte viability

Regarding nuclear maturation as viability parameter, 270 viable oocytes were obtained in three replicates and distributed among groups (CONT FRESH n=54; CONT VIT n=70; STC/VIT n=76; VIT/STC n=70). We observed that all vitrified groups presented lower MII rate percentages compared to CONT FRSH group, demonstrating the negative effect of vitrification on meiotic progression. Among vitrified groups CONT VIT did not differ from the other groups ($P > 0.05$), as demonstrated in Fig. 1A.

As for actin filaments integrity analysis, 238 viable oocytes were obtained in two replicates and distributed among groups (CONT FRESH n=47; CONT VIT n=68; STC/VIT n=73; VIT/STC n=50). We observed that all vitrified groups presented lower stained (intact) oocytes pattern compared to CONT FRESH group, demonstrating the negative effect of vitrification on actin filaments integrity. Among vitrified groups, the one submitted to STC before vitrification was the only one whose intact pattern was higher than non-stained (injured) pattern (Fig. 1B and C), suggesting that STC has positive effects in protecting actin filaments integrity from the negative effects of vitrification procedure.

In the gap juntions activity analyses, 404 viable oocytes were obtained in three replicates and distributed among groups (groups IMMATURE n=89; VIT n=122; STC n=89; STC/VIT n=104). We observed an increase in fluorescence intensity in all experimental groups compared to IMMATURE (IMMATURE 1.0 ± 0.49; VIT 1.76 ± 1.13; STC 1.38 ± 0.95; STC/VIT 1.58 ± 0.99; Means ± SD; $P < 0.05$), as demonstrated in Fig. 1D and F. We also evaluated the staining pattern of each oocyte (High/Middle/Low). In this analysis, IMMATURE group presented similar percentages of Middle and Low patterns. Taken IMMATURE group as the expected pattern, we observed that VIT group displayed a different pattern ($p < 0.05$), suggesting a detrimental effect when vitrification is applied. Witch appeared to be attenuated when STC is performed before vitrification, since STC/VIT group and IMMATURE group presented similar pattern ($p > 0.05$), as demonstrated in Fig. 1E.

3.2. Assay 2: Embryo production

For analysis, 827 viable oocytes were obtained in five replicates and distributed in three experimental groups (groups SPOM: n=186; STC/

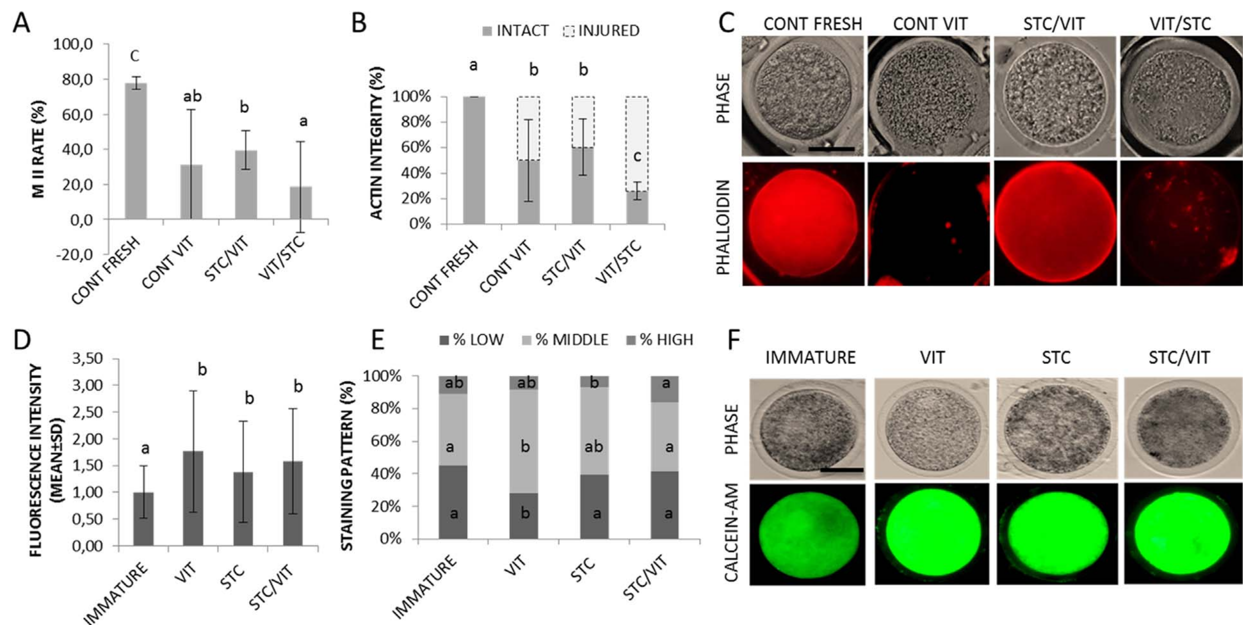

Fig. 1. Effect of STC with cAMP modulator before and after vitrification on oocyte viability. In assay 1, viable oocytes were distributed among groups CONT FRESH, CONT VIT, STC/VIT and VIT/STC, for nuclear maturation (n=270) and actin filaments integrity (n=238) analysis. For Gap junctions activity 404 oocytes were distributed among IMMATURE, VIT, STC and STC/VIT groups. (A) Graph display oocyte matured (M II) rate; (B) Graph display oocytes actin filaments integrity patterns (injured/intact); (C and F) Representative images of experimental groups. Scale bar 100 µm; (D) Graph display fluorescence intensity related to gap juntions activity; (E) Graph display staining patterns related to gap juntions activity. Graphs A and B display bars indicating the variation associated to replicates. Superscript letters denote statistical difference ($p < 0.05$) between groups.

Table 1
Embryo *in vitro* production of vitrified-warmed oocytes before and after STC with cAMP modulators.

Groups	N viable oocytes	Cleavage rate	Blastocyst rate		
		n (%)	N	% (cleavage)	% (viable oocytes)
SPOM	186	137 (73.66)^{a}	43	34.66^{a}	25.14^{a}
STC/VIT	316	25 (7.91)^{b}	3	10.71^{ab}	1.34^{b}
VIT/STC	325	15 (4.62)^{b}	0	0.00^{b}	0.00^{b}

For analysis, 827 viable oocytes were obtained in five replicates and distributed in three experimental groups (SPOM, STC/VIT and VIT/STC). Table display cleavage and blastocyst rate between groups. Superscript letters denote statistical difference ($p < 0.05$, chi-square test-χ2) between groups.

VIT: n=316; and VIT/STC: n=325), before IVF and IVC. We observed that vitrified groups display a significant reduction in cleavage rate (SPOM: 73.66^{a}; STC/VIT: 7.91^{b}; and VIT/STC: 4.62^{b}), as presented en Table 1. As for blastocyst production, none of the 325 viable oocytes of the VIT/STC group develops into a blastocyst stage. So taken in consideration the cleaved zygotes, only the VIT/STC differ from control group (SPOM: 34.66^{a}; STC/VIT: 10.71^{ab}; and VIT/STC: 0.00^{b}), and considering the viable oocytes submitted to treatments both vitrified groups had lower blastocyst rate (SPOM: 25.14^{a}; STC/VIT: 1.34^{b}; and VIT/STC: 0.00^{b}).

4. Discussion

The main findings of this study were that: (i) short term culture with cAMP modulators significantly improves cytoskeletal actin filaments integrity of vitrified-warmed bovine oocytes; (ii) vitrification, as well as STC, caused an increase in gap junctions activity, but immature vitrified oocytes differ from the expected staining pattern, effect that was attenuated when the STC was performed prior to vitrification; and (iii) vitrification significantly impaired cleavage and embryo production, regardless the STC treatment.

Vitrified oocytes viability is often assessed by morphological evaluation and fertilization competence. Such parameters provide limited information about the cryoinjury in oocytes undergoing vitrification (Coticchio et al., 2005, 2007), therefore little is known about how to prevent or reverse these effects. Thus, the present study evaluated different cellular parameters such as nuclear maturation progression, cytoskeletal actin filaments integrity and gap junctions activity besides the cleavage and embryo production in order to compare the effect of a STC with cAMP modulators in vitrified oocytes viability.

Our results demonstrate a decrease in matured oocytes rate in vitrified groups as a result of a deleterious effect of vitrification on meiosis resumption competence that leads to a significant decrease in viability of cryopreserved oocytes (Carroll et al., 1990; Mavrides and Morroll, 2005). Still, all vitrified groups presented lower percentage of oocytes displaying intact cytoskeleton. Phalloidin stain has a strong affinity with polymerized actin (F-actin), so the injured pattern (non-stained) results from the actin filaments depolymerization, possibly caused by vitrification process. Thereby confirming the deleterious effect of vitrification process on cytoskeleton integrity (Prentice et al., 2011).

In the present study, our approach was to vitrify immature oocytes, in order to overcome the disruption of the meiotic spindle by depolymerization of microtubules that occurs when MII oocytes are vitrified (Men et al., 2002; Rojas et al., 2004; Prentice et al., 2011). However, as our results showed, even with the vitrification of immature oocytes nuclear maturation was impaired. This happens because immature oocytes presents lower permeability of the plasma membrane to cryoprotectants (Agca et al., 1998), and the damage to the cytoskeleton after rewarming affects the segregation of chromosomes in meiosis I phase (Saragusty and Arav, 2011) – a cytoskeleton dependent event (Li and Albertini, 2013). Together, those results demonstrate the importance of cytoskeleton on nuclear maturation events, and also demonstrates the impact of vitrification procedure.

Regarding the analysis of nuclear maturation, among vitrified groups, STC before (STC/VIT) or after (VIT/STC) the vitrification had a similar result to the control group (CONT VIT). However, we observed a high deviation between repetitions in the CONT VIT (31.4% ± 31.2) and VIT/STC (18.6% ± 25.9) groups, compared to that found in

the STC/VIT group (39.5% ± 11.1). This finding may indicate that treatment with STC prior to vitrification has a more stable outcome with regard to meiotic progression. Moreover, our results demonstrated that the STC with cAMP modulators (the first step of SPOM system) applied before vitrification improved oocyte cryotolerance, as indicated by an increased in cytoskeletal actin filaments integrity, which has an important role in meiosis progression.

Several studies have demonstrated that higher cAMP levels before IVM improves oocyte competence and subsequent embryonic development of human (Shu et al., 2008; Zeng et al., 2013), murine (Albuz et al., 2010; Zeng et al., 2013), bovine (Luciano et al., 1999; Guixue et al., 2001; Albuz et al., 2010), ovine (Rose et al., 2013) and porcine (Funahashi et al., 1997) IVM oocytes.

Previous studies have hypothesized that a culture before IVM inhibiting the meiotic progression with Roscovitin, might offer to oocytes conditions to acquire greater developmental conditions after vitrification-warming, but these pretreatment did not return beneficial effects on vitrified oocyte viability (Albarracín et al., 2005; Diez et al., 2005). It seems like Roscovitin might induce significant changes, such as disruption of the integrity of the surrounding cumulus cells, swelling of the mitochondrial crista and degeneration of the cortical granules (Lonergan et al., 2003). Therefore, it is crucial to identify substances that can modulate cAMP levels without side effects on oocytes. In supplementary data we demonstrated that those cAMP modulators, FK and IBMX, do not cause detrimental effect on oocytes.

A recent study has showed that the addition of FSK or IBMX to the IVM medium tended to improve the developmental competence after IVF of vitrified-warmed GV oocytes (Ezoe et al., 2015). In our study, a STC with FK and IBMX after oocytes vitrification-warming did not return benefits regarding the viability parameters assessed - only when STC was performed prior to vitrification/warming benefits were observed.

It is known that cryopreservation causes damage to gap junctions, interrupting communication between the oocyte and cumulus cells (Diez et al., 2005). Since STC with cAMP modulators have been proven to improve gap junctions activity (Albuz et al., 2010), gap junctions were also a parameter assessed in this study. Unexpectedly, vitrification procedure itself also increased gap junctional activity, as was found for STC procedure. However, concerning staining pattern analysis, our results showed only vitrified group was different from immature oocytes taken as expected pattern, suggesting vitrified and STC groups could have gap activity affected by different ways.

It has been demonstrated that matured oocytes exposed to cryoprotectants and temperature drop during cryopreservation exhibited a transient increase in intracellular free calcium (Mattioli et al., 2003), which results in blocking the sperm entry by early release of cortical granules, a calcium-dependent event (Kline and Kline, 1992), thereby reducing the competence for development of oocytes. Besides gap junctional activity, calcein AM assay, used in this study, provides an intracellular free calcium estimative, so it is possible that this increase of calcium during the vitrification interfered with measurements of cumulus-oocyte communication. Additionally previous studies have reported that a transient increase of free calcium it's an environmental change that may lead in spontaneous activation (SA), which is an incomplete and abortive activation that severely damage oocyte developmental competence (Cui et al., 2013).

Regarding the increase in fluorescence intensity due to STC, it is likely to be involved with the calcium role in oocyte maturation (Homa, 1995; He et al., 1997). In many cell types, the activation of hormones and growth factors receptors causes an increase in intracellular free calcium (reviewed by (Williamson and Monck, 1990)). A calcium rise was described in bovine COCs after their exposure to IVM hormones (Silvestre et al., 2012). *In vivo,* the LH surge causes a rise in calcium free that starts in cumulus cells (Gudermann et al., 1992; Mattioli et al., 1998). Stimulation of LHRs leads to activation of two intracellular signaling pathways. The first refers to the activation of adenylyl cyclase, resulting in a transient increase in cAMP (Eppig and Downs, 1984). The second involves the stimulation of phospholipase C and production of inositol phosphates, most specifically 1,4,5-inositol triphosphate (IP3), which leads to elevation in calcium levels (Gudermann et al., 1992), resulting in a decrease in cAMP through calcium-activated phosphodiesterases (Bornslaeger et al., 1984). The calcium is provided, besides the intracellular stocks, from cumulus cells, throw gap junctions (Carroll and Swann, 1992), throw plasma membrane channels (Murnane and DeFelice, 1993; Tosti et al., 2000). So, the increase in cAMP mediated by Forskolin stimulation may activate the downstream degradation pathway, elevating both PDE C and calcium, but the IBMX inhibition let calcium free to bind calcein. Still, STC prolongs gap junctions communications (Albuz et al., 2010), explaining an important source of calcium and its increased levels.

Additionally, our data regarding the embryo production shows that vitrification significantly impaired cleavage and development of blastocysts, regardless the STC treatment. Curiously, the group submitted to STC before vitrification (STC/VIT) display similar cleavage rate compared to control group (p=0.0549), however we believe that it is a really small probability of those groups to be similar, which may indicate a tendency of impaired embryo development showed in this group. As that group presents a small number of cleaved structures, this tendency it is not evident in that experiment. Results also demonstrate that, regarding the blastocyst rate (considering viable oocytes submitted to treatments), the vitrified groups are similar (p=0.1192). Although mathematically the probability to form zero or three blastocyst from 325/316 viable oocytes (VIT/STC and STC/VIT groups, respectively) are the same, considering this large amount of 5 repetitions with more than 600 biological structures, only the STC/VIT group was able to give conditions to develop blastocysts.

It is noteworthy that if only embryo production experiments were taken, the STC before vitrification of immature oocytes would not be considered in cryopreservation programs. However, in the present study we also consider oocyte quality parameters after treatment, which showed benefits on maturation. Therefore, we can conclude that STC with cAMP modulators, Forskolin and IBMX, decreases cytoskeleton injuries caused by oocyte vitrification, that might increase oocyte viability and meiosis resumption, however we suggest that further studies taken into consideration further improvements on that system, favoring embryo development.

Conflict of interest statement

All authors declare that this manuscript has not been published or simultaneously submitted for publication elsewhere. Authors also declare that this scientific work was conducted following the ethical guidelines of research and there is no conflict of interest.

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Acknowledgements

This study was supported by Foundation for Research of the State of Rio de Janeiro (FAPERJ) and scholarship granted by the Higher Education Personnel Improvement Coordination (CAPES).

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.livsci.2017.01.013.

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