Role of cumulus cells during vitrification and fertilization of mature bovine oocytes: Effects on survival, fertilization, and blastocyst development

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Role of cumulus cells during vitrification and fertilization of mature bovine oocytes; effects on survival, fertilization, and blastocyst development

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A B S T R A C T

This study was designed to determine the role of cumulus cells during vitrification of bovine oocytes. Mature cumulus oocytes complexes with many layers of cumulus cells (COCs), corona radiata oocytes, with two layers of cumulus cells (CRs) and denuded oocytes without cumulus cells (DOs) were vitrified in 15% ethylene glycol (EG), 15% dimethylsulfoxide (DMSO) and 0.5M sucrose. Oocytes that survived the vitrification process were fertilized. Denuded oocytes were fertilized with or without supplementation of intact COCs (DOsCOCs). First, survival and embryo development rates were studied. Higher survival rates were obtained for DOs and DOsCOCs (94% and 95% respectively) compared with COCs (82.7%, $P<0.05$). Corona radiata oocytes showed similar survival rates when compared with denuded oocytes. The cleavage and blastocyst rates of vitrified DOs were compromised, since cumulus cells were not present during the fertilization (34% and 2.7% respectively). However, the situation could be reverted when DOs were supplemented with intact COCs (DOsCOCs) (62.7% and 12.7% respectively, $P<0.05$). Vitrified CRs showed similar cleavage and blastocyst rate compared with COCs (49.3% vs.54.8% and 7.7% vs. 4.9% respectively). In the second experiment, the penetration rate was analyzed. Removing cumulus cells before fertilization reduced the fertilization of vitrified DOs compared with COCs (24.3% vs.52.8%, $P<0.05$). The supplementation of DOs with intact COCs (DOsCOCs) improved the fertilization rate though (49.6%, $P<0.05$). No differences in the fertilization rate were found between CRs and COCs. In the third experiment, parthenogenetic activation was examined. Interestingly, the CRs group showed higher cleavage and blastocyst rates than the COCs (76.8% vs. 39.1% and 29.6% vs. 7.5 %respectively, $P<0.05$). Furthermore, oocytes from vitrified CRs had the same odds to become a blastocyst as fresh oocytes (1.1 vs. 1.5, respectively).
In conclusion, our data demonstrated that cumulus cells reduce survival after the vitrification of mature bovine oocytes. Since cumulus cells are required for fertilization, the use of partially denuded oocytes (CRs) or the addition of intact COCs (DOsCOCs) during fertilization can result in higher survival and embryo development after vitrification.
Keywords: Bovine, oocyte, vitrification, cumulus cells

1. Introduction

The vitrification of oocytes provides many benefits for assisted reproductive technology (ART). In humans, it allows to reduce the number of embryos produced at any given time, it permits synchronization in donor-recipient programs and it preserves the fertility of young women receiving cancer treatment; in animals, it allows preserving genetic diversity and it increases the material available for research and animal breeding programs [1, 2]. Oocytes are very sensitive to vitrification because of their high lipid content and low surface-to-volume ratio. Furthermore, their complex structure (zona pellucida, oolemma, cortical granules, metaphase plate or germinal vesicle) can be severely damaged during cooling and warming [3]. Oocyte vitrification can induce rupture of the oolemma, distortion of the metaphase plate in the mature oocyte, and premature extrusion of the cortical granules leading zona hardening, all of which prevent normal fertilization of the vitrified-warmed oocytes [4]. Human ART has circumvented these problems and laboratories have reported obtaining blastocyst formation and live births from vitrified oocytes at rates equivalent to those from fresh oocytes[5] . However, the development rates of vitrified-warmed bovine oocytes remain low in comparison with their fresh counterparts, since bovine oocytes are more sensitive to chilling because they contain high amounts of lipids [6].

The success of oocyte vitrification depends on many factors that have been studied for several years[7]. These factors can be divided in two groups: oocyte factors, referring to the presence of cumulus cells or the developmental stage of the oocyte (mature or germinal vesicle) and technical factors, referring to different protocols, cryoprotectants and devices used. Although most of these factors have been studied in the last decade, some of them, such as the effect of cumulus cells during vitrification still remain unclear. In cattle, it is known that the presence of cumulus cells is necessary for a correct maturation, fertilization and subsequently embryo development [8, 9]. However, it is still controversial if the presence of cumulus cells is beneficial during vitrification of bovine mature oocytes [10-12]. It has been suggested that cumulus cells may protect against cryo-injury during vitrification by minimizing the release of cortical granules, thus preventing premature zona hardening [13]. On the other hand, the presence of cumulus cells during cryopreservation could limit the exchange of water and CPAs, which could cause inadequate dehydration and/or CPA entry and consecutive ice crystal formation, which leads to an inappropriate protection of human and mouse oocytes [14] . In cattle, Zhou et al. showed [12] that vitrified partially denuded immature oocytes develop at significantly lower rates than vitrified cumulus enclosed oocytes. However, no significant differences were detected when mature oocytes were partially denuded before vitrification. These authors suggest that cumulus enclosed and partially denuded mature oocytes display the same survival, cleavage and blastocyst rates after vitrification, probably due to the fact that cumulus cells were detrimental during vitrification, compromising the benefits of cumulus cells during IVF. The aim of our study was to further determine the possible detrimental or beneficial effect of cumulus cells during the vitrification of mature bovine oocytes. In particular, we analyzed whether an intact, partially removed or completely removed cumulus, or the mere presence of cumulus cells, affects survival, fertilization and subsequent embryo development of vitrified and fresh mature bovine oocytes. To disentangle effects on fertilization and development respectively, we also studied embryo development after parthenogenetic activation.

2. Material and Methods

2.1. Media and reagents

Basic Eagle's Medium, Tissue Culture medium (TCM) 199, Minimal Essential Medium non-essential amino acids, kanamycin, and gentamycin were purchased from Life Technologies Europe and all other components were obtained from Sigma (Bornem, Belgium).

2.2. Collection and in vitro maturation of oocytes

Bovine ovaries obtained from a local slaughterhouse were rinsed twice in physiological saline supplemented with kanamycin (25mg/ml). Cumulus-oocyte complexes (COCs) were aspirated from 2-8 mm follicles with an 18 gauge needle attached to 10 ml syringe and matured in groups of 60 oocytes in TCM199 supplemented with 50 mg/ml gentamycin and 20ng/ml epidermal growth factor for 22 h at 38.5°C in 5% CO_2 in air.

2.3. Vitrification and Warming

Matured oocytes were vitrified as described by Kuwayama and colleagues [15] with some modifications. The handling medium (HM) used was TCM199/ Hank's/ Hepes supplemented with 20% Fetal Bovine Serum (FBS, Greiner Bio-one). All the vitrification media were prepared using this HM. Vitrification and warming steps were performed at 39°C on a heated plate.

Vitrification was performed in two steps: equilibration and vitrification. Oocytes were equilibrated by transferring them sequentially in three drops of 75 µl of equilibration solution (ES) composed of HM with 7.5% ethylene glycol (EG) and 7.5% dimethyl-sulfoxide (DMSO). After oocytes regained their original volume, they were subsequently transferred into four consecutive 50 µl drops of vitrification solution (VS) composed of HM with 15% EG, 15% DMSO and 0.5 M sucrose. Oocytes were exposed to equilibration solution for 10-15 min and to vitrification solution for 45-60 sec. Four oocytes were loaded to a manufactured-cryotop and within 5 sec submerged in liquid nitrogen (LN_2). After one week in LN_2, oocytes were warmed by transferring them to a warming solution composed of HM with 1M of sucrose. This was followed by a three step wash-out of the hyperosmolar sucrose reduced from 1M to 0.5M (washing 1, 3 min), 0.25M (washing 2, 5 min) and 0 M in HM (washing 3, 5 min). Oocytes were washed in HM three times and then incubated in maturation medium for 2 hours to allow them to recover.

2.4. Fertilization and culture

Fresh (non-vitrified) and vitrified oocytes were fertilized in the same conditions. Frozen-thawed bull spermatozoa were separated using a Percoll gradient (45 % and 90 %; Pharmacia, GE Healthcare). The final sperm concentration of $1x10^6$spermatozoa/mL was adjusted in IVF Tyrode's albumin-pyruvate–lactate (TALP), consisting of bicarbonate-buffered Tyrode solution, supplemented with BSA (6mg/ml) and heparin (25 mg/ml).

At 21 h post-insemination, presumptive zygotes were vortexed to remove cumulus cells, washed and cultured in groups of 25 in 50 µl droplets of synthetic oviductal fluid medium (SOF) supplemented with ITS (5 µg/ml Insulin + 5 µg/ml Transferrin + 5 ng/ml Selenium) and 0.4% BSA. Culture occurred at 38.5º C in 5% CO_2, 5% O_2 and 90% N_2. Cleavage rates were determined after 48h post insemination and blastocyst rate after 8 days post insemination.

2.5. Parthenogenetic activation

Oocytes were incubated in 5 µM ionomycin for 5 min. Next, they were incubated in SOF medium supplemented with 2 mM 6-dimethyl-aminopurine (6DMAP) for 4 hours. After incubation, presumptive parthenotes were washed twice and cultured in SOF medium in the same conditions as fertilized oocytes. Cleavage rates were determined at 48h post activation and blastocyst rate at 8 days post activation.

2.6. Assessment of survival and penetration rate

After fertilization, oocyte survival was evaluated morphologically. The criteria used to classify oocytes as surviving or degenerated have been described elsewhere [11]. Briefly, oocytes with intact oolemma, intact zona pellucida and homogenous and dark cytoplasm are considered as surviving oocytes. Only surviving oocytes were used for in vitro culture.

Fertilization rate was determined with the nuclear staining Hoechst 33342 (Molecular Probes, Invitrogen, Merelbeke, Belgium) which selectively binds to double stranded DNA. Presumed zygotes were fixed in 4% formalin and then stained with Hoechst 10 µg/ml for 10 min. Successful fertilization was characterized by the presence of two pronuclei and three or more pronuclei were considered as indicative for polyspermy.

2.7. Experimental design

In experiment 1 (n=1161), mature oocytes were randomly divided into two groups; fresh and vitrified. Fresh oocytes were subdivided into four groups: cumulus complex oocytes (Fresh COCs), corona radiata oocytes (Fresh CRs), denuded oocytes (Fresh DOs) and denuded oocytes supplemented with intact COCs during fertilization (Fresh DOsCOCs). Oocytes in the vitrified group were vitrified as COCs, CRs or DOs and subdivided into four groups after warming; cumulus complex oocytes (Vitrified COCs), corona radiata oocytes (Vitrified CRs), denuded oocytes (Vitrified DOs), and denuded oocytes supplemented with fresh COCs during fertilization (Vitrified DOsCOCs) (figure 1). CRs and DOs were partially or completely denuded by gently pipetting in HM.

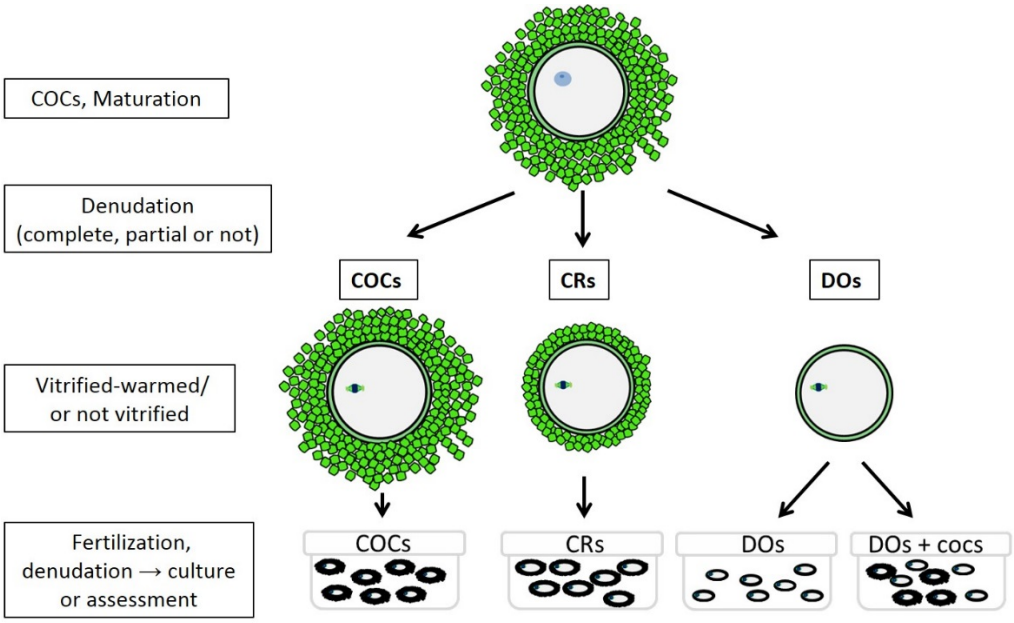

Fig. 1. Experimental design of experiment 1 and 2. In vitro matured cumulus-oocyte complexes (COCs) were randomly assigned to three groups: COCs, corona radiata oocytes (CRs), and denuded oocytes (DOs). CRs and DOs were created by partial or complete removal of the cumulus cells. Then, oocytes in all three groups were randomly assigned to be either vitrified and warmed, or not vitrified. The vitrified and not-vitrified DOs were further subdivided in DOs and DOs supplemented with not-vitrified COCs (DOs + COCs). The, thus, obtained four vitrified and four not-vitrified groups were fertilized in vitro followed by embryo culture (experiment 1) or assessment of fertilization (experiment 2).

Intact COCs which were supplemented to vitrified oocytes during fertilization were obtained one day before warming and matured in the same conditions as the rest of the oocytes. These supplemented COCs were removed by pipetting after fertilization and before assessing survival, cleavage and blastocyst rates. In experiment 2 (n= 1073), the experimental design was similar, except that presumptive zygotes were not cultured, but were denuded, fixed and stained to study the fertilization rate. In experiment 3 (n= 601), mature oocytes were randomly divided in four groups; fresh COCs, vitrified COCs, vitrified CRs and vitrified DOs. Parthenotes were produced by parthenogenetic activation and cleavage and blastocyst rates were assed.

2.8. Statistical analysis

Several binary logistic regression models were fit to determine the role of cumulus cells and the impact of the treatment on the likelihood of survival, embryo development, fertilization and polyspermy in experiment 1 and 2, and on the likelihood of parthenote development in experiment 3, using SPSS statistics version 22. The models included the likelihood of survival, embryo development, fertilization, and polyspermy and parthenote development respectively, as binary outcome variables and the oocyte group (COCs, CRs, DOs, and DOsCOCs), the treatment (fresh and vitrified), and the interaction term between the oocyte group and treatment as categorical independent variables. Six replicates were performed for experiment 1, five for experiment 2 and four for experiment 3. For all outcome variables, the replicate was forced in the model to account for clustering of observations within a replicate. Furthermore, each vitrified oocyte was compared with its respective fresh counterparts. A Bonferroni´s correction was applied to correct for multiple comparisons. Statistical significance was assessed at P< 0.05.

3. Results

3.1. Experiment 1

Survival rates of fresh groups were all very close to 100%, with no significant differences between groups (figure 2). However, vitrified denuded oocytes in the groups DOs and DOsCOCs presented higher survival rates than vitrified COCs (94% and 95% vs. 82.7% respectively, P<0.05). Survival rates of vitrified CRs (86.2%) did not differ significantly from those of vitrified denuded oocytes. Vitrified COCs and CRs had significantly lower survival rates compared with their fresh counterparts (82.7% and 86.2% vs.98 % and 98.6% respectively, P<0.05).

Removing cumulus cells after maturation significantly decreased the number of oocytes that cleaved (P<0.05) (figure 3). However, an increase of the cleavage rate was found in DOsCOCs compared with DOs (68.5 % vs. 53.7% respectively, P<0.05). The same effect was observed among the vitrified groups (P<0.05). Furthermore, the addition of COCs to vitrified DOs (DOsCOCs) resulted in cleavage rates comparable with those in vitrified COCs (62.8% vs. 54.9%, respectively, P>0.05). Cleavage rates of vitrified COCs (54.9%) and DOs (34%) were significantly lower than fresh COCs (82.7%) and fresh DOs (53.6%) (P<0.05).

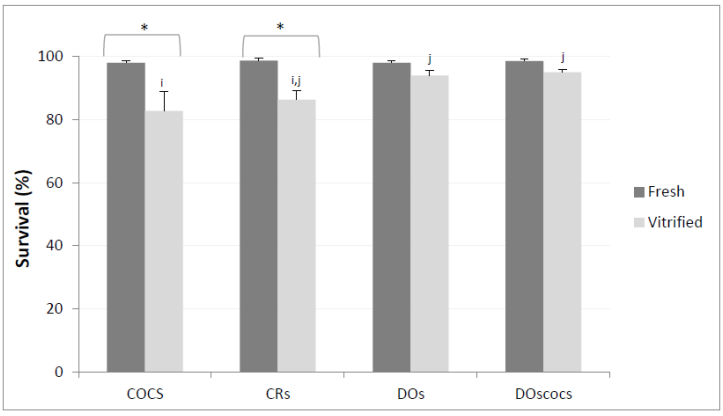

Fig. 2. Survival of control and vitrified mature bovine oocytes. Mean ± SEM, n = 1161. [i,j]Different superscripts indicate significant differences between the oocyte groups for the vitrified oocytes; *Indication of significant differences between each control with its respective vitrified group. Significance was assessed at P < 0.05. COCs, cumulus complex oocytes; CRs, corona radiata oocytes; DOs, denuded oocytes; DOsCOCs, denuded oocytes supplemented with cumulus complex oocytes.

Fresh oocytes had a higher chance of becoming blastocysts compared with vitrified oocytes, with the exception of DOs, for which blastocysts rate was not significantly different between vitrified and fresh oocytes. Removing cumulus cells among fresh groups, decreased the blastocyst development for CRs (20%) and DOs (9.3%) compared with COCs (42%), P<0.05. However, the supplementation of the intact COCs to DOs (DOsCOCs) increased the blastocyst development to rates comparable with those of COCs (26.7% vs. 42% respectively). Among vitrified groups, we observed that removing the cumulus cells did not have a significant effect on the blastocyst development of CR (7.7%) and Dos (2.7%) compared with COCs (4.9%), but the blastocyst rate of DOsCOCs was significantly higher compared with DOs (12.7% vs. 2.7% respectively, P<0.05).

3.2. Experiment 2

Removing cumulus cells before fertilization appeared to have a negative effect on fertilization rates (figure 4). For fresh oocytes, fertilization rates of CRs (39.5%) and DOs (24.7%) were significantly lower (P<0.05) than that of COCs (71.6%). The supplementation of intact COCs to DOs could revert the fertilization rate, being not significantly different from COCs (59.3% vs. 71.6% respectively, P>0.05). We observed the same effect in vitrified oocytes. Fertilization rate was significantly higher for COCs compared with DOs (52.8% vs. 24.3% respectively, P<0.05), and the situation could be reverted when DOs were supplemented with intact COCs (DOsCOCs) (49.6%, P>0.05). Only vitrified COCs showed a fertilization rate which was significantly different from that in their fresh counterparts (71.6% vs. 52.8%, P<0.05). Polyspermy rates (figure 4) were also numerically lower in DOs and CRs compared with COCs and DOsCOCs, but none of the differences between groups were significant.

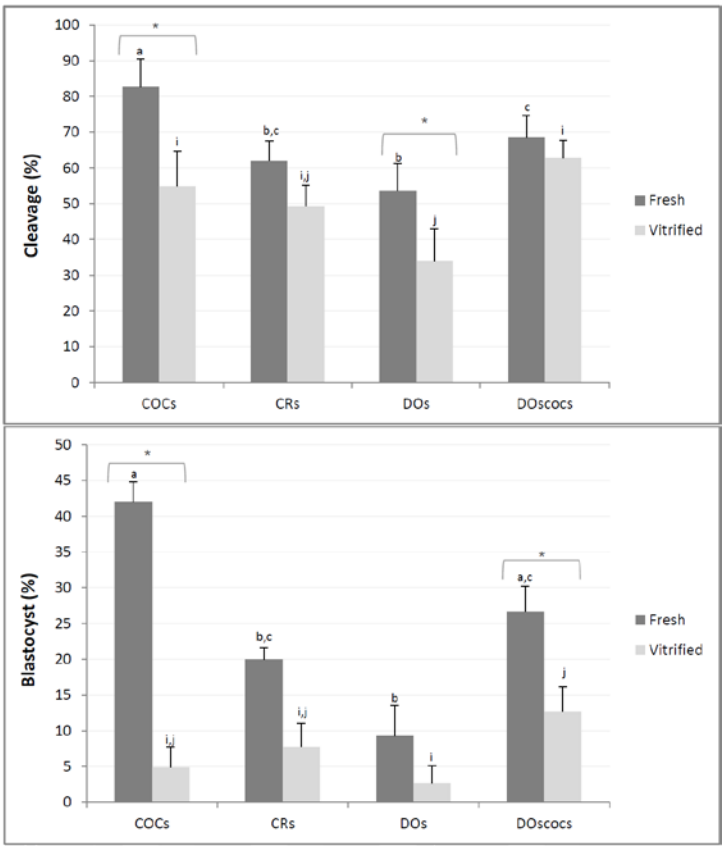

Fig. 3. Cleavage and blastocyst development of control and vitrified mature bovine oocytes after in vitro production. Data are given as mean ± SEM (n = 1161, six replicates). [a,b,c]Different superscripts indicate significant differences between the oocyte groups for the control group; [i,j]Different superscripts indicate significant differences between the oocyte groups for the vitrified group; *Indication of significant differences between each control with its respective vitrified group. Significance was assessed at P < 0.05. COCs, cumulus complex oocytes; CRs, corona radiata oocytes; DOs, denuded oocytes; DOsCOCs, denuded oocytes supplemented with cumulus complex oocytes.

3.3. Experiment 3

In figure 5, we observed that fresh groups had higher chances to cleave after parthenogenetic activation when compared with vitrified groups (P<0.05). Among the vitrified groups, cleavages rates of CRs were significantly higher compared with those of COCs (76.8% vs.39.1% respectively, P<0.05). Blastocyst development of vitrified CRs (29.6%) was comparable with that of fresh oocytes (41.9%), and significantly higher than that of COCs (7.5%, P<0.05).

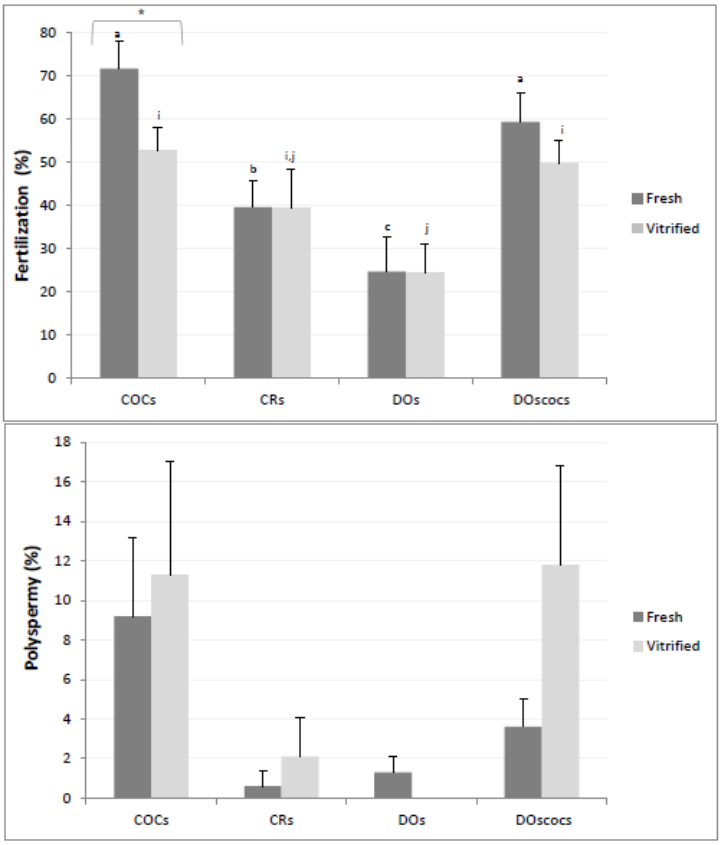

Fig. 4. Fertilization rates of control and vitrified mature oocytes. Data are given as mean þ SEM, n ¼ 1073, five replicates. [a,b,c]Different superscripts indicate significant differences between the oocyte groups for the control group; i,jDifferent superscripts indicate significant differences between the oocyte groups for the vitrified group; *Indication of significant differences between each control with its respective vitrified group. Significance was assessed at P < 0.05. COCs, cumulus complex oocytes; CRs, corona radiate oocytes; DOs, denuded oocytes; DOsCOCs, denuded oocytes supplemented with cumulus complex oocytes.

4. Discussion

The effect of cumulus cells during vitrification has been studied for many years, but discrepant results have been found in several species. In human, a beneficial effect of cumulus cells in the survival of mature oocytes was observed at first [15, 16], however Minasi and colleagues [17] reported no differences in the survival of COCs compared with denuded oocytes. In equine, the presence of cumulus cells was found to be beneficial during the vitrification of mature oocytes [18], whilst they were reported to play a detrimental role during the vitrification of buffalo oocytes [19]. On the other hand, no differences were found in the survival rate between cumulus complex oocytes and denuded ovine oocytes [20] . In cattle, discrepant results have been reported. Dinnyes and colleagues [10] did not observe any effect when cumulus cells were present during vitrification of bovine oocytes. Similarly, Zhou and colleagues [12] did not observe any effect in the survival of mature bovine oocytes when they were vitrified with cumulus cells. Nevertheless, Chian and colleagues [11] observed that cumulus cells have a detrimental effect on the survival rate of vitrified mature oocytes. Our observations agree with the latter study. We observed that oocytes surrounded by cumulus cells such as oocytes in the COCs or CRs groups have less chance to survive compared with their fresh counterparts, whilst denuded oocytes presented the

same survival rate as their fresh counterparts. According to the results observed in our study, we think discrepancies with previous reports were due to the fact that we analyzed the survival of COCs, CRs and DOs, whereas Zhou and colleagues [12] studied the survival of COCs and partially denuded oocytes. Those partially denuded oocytes could be considered as a different group than we used in the current study, since they had a different amount of cumulus cells with respect to CRs and DOs. We hypothesize that communication between the cumulus cells and the oocyte via gap junction and connexin hemichannels not incorporated into gap junctions results in inhomogeneous shrinking, damaging the structure of the oocyte. Hemichannels are normally closed, but they can be opened under stress conditions, as the vitrification process. Open hemichannels allow the movement of the water and some cryoprotectants, leading to inhomogeneous shrinking of the oocyte. In addition, cumulus cells can also hinder the diffusion of water and CPAs, resulting in an inadequate cell protection. It is also remarkable that denuded or corona radiata oocytes were easier to handle when a manufactured cryotop was used during vitrification. Moreover, the amount of liquid surrounding these oocytes was lower compared with COCs. A lower amount of liquid surrounding the oocyte leads to a higher cooling and warming rate [21], which could be positive for survival of the CRs and DOs compared with COCs.

It is known that removal of cumulus cells shortly before in vitro fertilization strongly decreases fertilization rates in cattle [8, 9]. During fertilization, cumulus cells attract, trap and select the spermatozoa [22, 23]. They are also important to induce sperm capacitation, acrosome reaction and penetration [22-25] and to prevent zona pellucida hardening [26, 27]. In our study, removal of cumulus cells after maturation decreased the fertilization, cleavage and blastocyst rates, as expected. To solve this problem, we supplemented DOs with intact COCs during the fertilization. In this way, DOs can restore their developmental capability [28]. Attanasio and colleagues [29] showed that the cleavage rate of vitrified buffalo oocytes increases when DOs are supplemented with intact COCs, but not the blastocyst rate. The data of the present study showed that cleavage and blastocyst rates can be improved in denuded vitrified bovine oocytes when they are supplemented with intact COCs.

Another way to solve the negative effect of cumulus cells during vitrification and to allow a positive influence during fertilization involves a partial reduction of cumulus cells before vitrification [30]. Therefore, in the present study, we included a CRs group, which represents the corona radiata consisting of only two or three layers of cumulus cells. We observed that the survival rate of CRs after vitrification was comparable with that in DOs, while the fertilization rate tended to be higher. Furthermore, vitrified CRs showed similar embryo development than COCs. On the other hand, fresh COCs displayed higher fertilization and subsequently embryo development than CRs.

It has been published that the exposition to low temperatures and cryoprotectants can lead to the release of cortical granules in oocytes, which can cause zona hardening [31]. In our study, this effect may have been small, as the differences in the fertilization rate between fresh and vitrified DOs supplemented with fresh COCs (DOsCOCs) were very similar and not significant. Furthermore, we observed no differences between fresh and vitrified oocytes in zona hardening (assessed by pronase digestion, data not shown). On the other hand, polyspermic penetration can be related to abnormalities in release and dispersal of cortical granules[32]. A proper cortical reaction was probably present, since we could not find significant differences in polyspermic penetration between vitrified and fresh oocytes.

To investigate the effect of cumulus cells during the vitrification of mature oocytes on embryo development, avoiding its confounding effect during fertilization, we performed parthenogenetic activation of vitrified mature oocytes after warming. We observed a lower developmental capacity of the vitrified COCs compared with the CRs. Interestingly, the CRs showed similar blastocyst rates as fresh oocytes. This suggests that partial removal of an excess of cumulus cells supports developmental competence of mature bovine oocytes after vitrification. This result disagrees with the data published by Dinnyes and colleagues [10], who found that the development following parthenogenetic activation was reduced in vitrified oocytes compared with fresh oocytes. Although the reduction of cumulus cells layers compromises the fertilization and subsequently embryo development, our parthenogenetic data proved that this was merely due to a reduced fertilization rate since parthenogenetic embryos could reach the blastocyst stage at high rates. Hence, the detrimental effect of cumulus cell removal can be mended by adding COCs during fertilization of vitrified warmed oocytes, as we showed here, or eventually by ICSI [4].

In conclusion, our data indicate that it is advisable to remove at least a part of the cumulus cells before the vitrification of mature bovine oocytes. Denuded oocytes survive vitrification at higher rates than COCs, although their fertilization and subsequent embryo development is compromised by the absence of cumulus cells. The supplementation of intact COCs can restore the situation, providing DOs with similar fertilization and embryo development rates as in vitrified COCs. On the other hand, although vitrified COCs have a higher fertilization rate, their survival and embryo development is compromised by the presence of cumulus cells. The use of corona radiata oocyte can overcome these two factors, improving the efficiency of vitrified mature bovine oocytes. However , if future research could show why COCs vitrify more poorly than DOs, perhaps the vitrification medium recipe or the procedures could be optimized for COCs to yield best survival combined with best fertilizing and developmental capacity.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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