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Cryotolerance of *in vitro* matured and vitrified bovine oocytes supplemented with L-carnitine and niacin#

K. Anjitha[1*], A. Amritha[2], R. S. Abhilash[2], C. Jayakumar[3] and T. Marykutty[4]
Department of Animal Reproduction, Gynaecology and Obstetrics
College of Veterinary and Animal Sciences, Mannuthy, Thrissur- 680 651
Kerala Veterinary and Animal Sciences University, Kerala, India.

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Abstract

The study was designed to compare the cryotolerance of in vitro matured bovine oocytes, supplemented with L- carnitine and niacin in maturation media, vitrified using cryoloop technique. Bovine ovaries were collected from the slaughterhouse and cumulus-oocyte complexes (COCs) were retrieved by follicular aspiration. Culture quality COCs were selected and randomly allocated to three different experimental groups. Group I oocytes (control), were subjected to in vitro maturation (IVM) in basic maturation media, while Group II and III oocytes (experimental groups) were matured in media supplemented with L- carnitine (0.6 mg/mL) and niacin (400 µM/mL), respectively. The mean per cent of cumulus cell expansion was 80.92 ±0.02, 79.18 ± 0.03 and 87.95 ±0.02 for Group I, II and III, respectively. A significantly high ($p < 0.05$) cumulus cell expansion could be observed in niacin treated group than L- carnitine, whereas a difference close to being statistically significant ($p = 0.057$) was noticed between niacin treated and control groups. The matured oocytes were subjected to cryoloopvitrification using dimethylsulfoxide (15%), ethylene glycol (15%) and sucrose (0.05M) as cryoprotectants. The mean survival rate observed in the study was 67.26 ± 0.05, 76.34 ± 0.05 and 79.68 ± 0.03 per cent for Group I, II and III, respectively. Shrinkage of cytoplasm was the most common type of cryodamage observed in all the three groups followed by cracked zona pellucida, abnormal shape and increased peri vitelline space. The survived oocytes

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1. *MVSc scholar*
2. *Assistant Professor,*
   Department of Animal Reproduction, Gynaecology and Obstetrics, CVAS, Mannuthy
3. *Associate Professor and Head (i/c),*
   Department of Animal Reproduction, Gynaecology and Obstetrics , CVAS, Mannuthy
4. *Assistant Professor, CASAGB, CVAS, Mannuthy*

**Corresponding author: anjithak94@gmail.com ,Ph: 8590793517*

were subjected to in vitro fertilisation with no significant difference between the groups in fertilisation rate. The present study revealed the beneficiary effect of supplementing niacin over L- carnitine in maturation media as it improved the maturation and survival rates of bovine oocytes.

Keywords: *Vitrification, in vitro maturation, cumulus cells, survival rate*

Assisted reproductive technology is an important tool to optimise superior cow genetics, which includes *in vitro* fertilisation and embryo transfer. The ideal season is one factor that affects the success rate of embryo transfer. Cryopreservation of oocytes and embryos increases scheduling flexibility of embryo transfer. However, compared to spermatozoa or embryos, bovine oocytes are incredibly challenging to cryopreserve due to their large size, low surface to volume ratio, high cytoplasmic lipid content and low hydraulic conductivity. Vitrification of oocytes involves rapidly cooling small volumes of highly viscous solution having high cryoprotectant concentrations, resulting in the development of a glass like state both inside and outside the cell and thereby preventing crystallisation. The developmental competency of bovine oocytes following vitrification is highly compromised due to the production of reactive oxygen species (ROS), high lipid content and variation in cooling rates which is affected by the volume of vitrification solution used. Hence the present study is aimed at assessing the cryotolerance of *in vitro* matured bovine oocytes supplemented with L- carnitine and niacin vitrified using cryoloop.

Materials and methods

Bovine ovaries of unknown reproductive status were collected from Corporation slaughter house, Kuriachira, Thrissur and Meat Technology unit, KVASU. Ovaries were collected and transported to the laboratory within two hours of slaughter in 0.9 per cent normal saline (NS) supplemented with penicillin (100,000 IU/L) and streptomycin (100 mg/L) maintained at 37 to 39°C. The follicles measuring between 2 to 8 mm diameter were aspirated in oocyte collection medium supplemented with three per cent bovine serum albumin (BSA). The cumulus oocytes complexes (COCs) were subjected to morphological evaluation and grading for further processing (Kakkassery *et al*., 2010). Good quality oocytes obtained in each trial was grouped into three different maturation media for *in vitro* maturation.

In vitro maturation of oocytes

A group of 10-20 COCs were transferred into two hour pre- equilibrated (38.5°C) 100 µL droplets of maturation medium. Basic maturation medium used for *in vitro* maturation of Group I oocytes consisted of TCM-199 (HEPES modified) supplemented with 50 ng/mL human FSH, 1 µg/mL oestradiol-17β, 0.25mM sodium pyruvate, 0.68 mM L-glutamine, 10 µg/mL gentamycin sulphate and 10 per cent FBS. The basic maturation media was supplemented with L-carnitine (0.6 mg/mL) and niacin (400 µM/mL) for *in vitro* maturation of Group II and Group III oocytes, respectively. The culture condition set for the study was 38.5°C in 95 per cent humidified atmosphere of 5 per cent CO_2. Maturation of oocytes was assessed based on the degree of cumulus cells expansion (Kobayashi *et al*., 1994).

Vitrification of oocytes using cryoloop

Matured oocytes in Group I, II and III were vitrified using cryoloop method. The matured oocytes were transferred to 100 µL droplets of holding medium (HM: TCM-199 + 20 % FBS) and partially denuded by gentle repeated pipetting. These oocytes were transferred to vitrification solution I (VSI) and vitrification solution II (VSII) serially as follows. In a 35 mm Petri-dish one drop each of VSI (TCM-199 + 20 % FBS + 7.5 % DMSO + 7.5 % EG) and VSII (TCM-199 + 20 % FBS + 15 % DMSO + 15 % EG + 0.5 M sucrose) were taken. A group of 4 or 5 matured oocytes were first transferred to VSI for a period of five minutes, followed by VSII for 45 to 60seconds. The oocytes were then transferred to cryoloop preloaded with VSII under stereozoom microscope. Without pause, the cryoloop was quickly placed directly into cryovial containing liquid nitrogen. The cryovials were stored in a cryocan at -196ºC for a minimum of one week.

Warming and stepwise removal of cryoprotectant

After one week of storage period, the cryoloop containing vitrified oocytes was transferred immediately into a 35 mm Petri- dish containing warming solution which consisted of holding medium (HM: TCM-199+ 20 % FBS) supplemented with 1.25 M sucrose prewarmed at 39°C for three minutes. The oocytes were then transferred serially to holding media supplemented with 1.25M, 0.5M, 0.25M, 0.125M sucrose for 30 seconds each and finally to HM without sucrose. The oocytes were then cultured at 38.5°C in a humidified atmosphere of five per cent CO_2 in air for three hours prior to being subjected to *in vitro* fertilisation. The survival rate of vitrified oocytes was determined as the number of rehydrated oocytes appearing morphologically normal out of total number of oocytes vitrified (Mavrides and Morroll, 2002). Morphologically non- viable oocytes were studied for cryodamage following vitrification.

In vitro fertilisation and culture of vitrified oocytes

The spermatozoa were prepared by swim up method for one hour as described by Parrish *et al*. (1985). After 18h of co-incubation with sperm in modified Tyrode's medium, the presumed zygotes were transferred into modified SOF medium for culture. After 48h of culture, oocytes were examined for fertilisation changes. The changes observed were cleavage, second polar body extrusion and penetration of sperm into cytoplasm.

Statistical analysis

The results obtained were statistically analyzed using SPSS version 24.0 software. Chi-square/ Fisher's exact test was used for groupwise comparison.

Results and discussion

The mean per cent of oocytes with cumulus cell expansion in different groups were 80.92 ±0.02, 79.18 ± 0.03 and 87.95 ±0.02 for Group I, II and III, respectively. A significantly high (p - 0.05) cumulus cell expansion rate could be observed in niacin treated group than L- carnitine group and a difference close to being statistically significant (p = 0.057) was noticed between niacin and control. However, no significant difference could be observed between L- carnitine and control. This observation was in accordance with Almubarak *et al*. (2021), who noticed a significantly higher cumulus expansion rate in porcine oocytes when IVM media was supplemented with 600 µM niacin. This could be due to the potent antioxidant activity and reduction in ROS production in matured oocytes treated with niacin (Kafi *et al*., 2019). Also, niacin could promote granulosa cell growth through cell apoptotic signaling pathways, cell arrest and oxidative stress pathways (Wang *et al*., 2018; Wang *et al*., 2020) and resulted in better developmental competency. Chankitisakul *et al*. (2013) reported that L- carnitine treatment during IVM did not affect the maturation of bovine oocytes, which was in accordance with present study.

The survival rates of vitrified oocytes were 67.26 ± 0.05, 76.34 ± 0.05 and 79.68 ± 0.03 for Group 1, 2 and 3, respectively. A higher survival rate with a trend towards significance (p = 0.069) could be observed in vitrified oocytes supplemented with niacin than control. However, no significant difference could be observed between L- carnitine and niacin supplemented oocytes and L- carnitine and control. The result was also comparable to the observations by Maclellan *et al*. (2002), who obtained a survival rate of 64- 84 per cent for cryoloopvitrification of equine oocytes. Contrary to the finding, Mavrides and Morroll (2002) obtained a substantially higher survival rate of 90.50 per cent following cryoloopvitrification of matured bovine oocytes using DMSO, EG, poly ethylene glycol and ficoll as CPA. Yang *et al*. (2008) obtained a higher rate of 92.50 per cent morphologically viable bovine oocytes following microdropvitrification. These findings indicated that the type and concentration of CPAs, species and type of cryodevices used were having an effect on the oocyte survival rate following vitrification.

Vitrification predisposes oocytes to low glutathione and high ROS concentration (Kelly *et al.,* 2005) and high intra cellular lipid

Table 1. Effect of supplementation of L carnitine and niacin on cumulus cell expansion, oocyte survival, nuclear maturation and fertilisation rate

Group	Cumulus cell expansion (%)	Oocyte survival (%)	Fertilisation rate (%)
Group I (n = 112)	$80.92^{b*} \pm 0.02$	$67.26^{b\#} \pm 0.05$	22.58
Group II (n = 138)	$79.18^{b} \pm 0.03$	$76.34^{ab} \pm 0.05$	22.22
Group III (n = 161)	$87.95^{a*} \pm 0.02$	$79.68^{a\#} \pm 0.03$	23.22

**Significant at p = 0.057*

 # Significant at p = 0.069

Means having different superscripts within a column differ significantly at 5% level

in oocytes leads to low survival rate (Romek *et al*., 2009). The improved survival rate in niacin treated group than non- treated oocytes could be attributed to the lipid modifying effect, anti-oxidant properties with reduction in MDA level in bovine oocytes subjected to vitrification (Brown *et al*., 2001; Block *et al*., 2013). The expression of the *ACACA* and *PNPLA2* genes, which were involved in lipid metabolism were upregulated in cumulus cells and oocytes matured in the presence of niacin (Wang *et al.,* 2020).

Among the different types of cryodamages observed, shrinkage of cytoplasm was most common than other cryodamages in the present study which might be due to the osmotic imbalance caused by the use of concentrated cryoprotectants (Men *et al*., 1997). Vitrification induced lipid to undergo a more packed configuration in the zona pellucida, which caused mechanical hardening and resulted in cracked zona pellucida. Other possible reasons may be the temperature difference between the outer layer and inner core of oocytes (Dhali *et al*., 2000), zona hardening induced by the cryoprotectant DMSO (Vincent *et al.,* 1990). Although non- significant, low cryodamage was observed in experimental groups than control which could be due to the anti-oxidant, as well as anti-apoptotic properties of both L- carnitine and niacin (Moaward *et al.,* 2013; El Sheikh *et al*., 2020)

The overall fertilisation rate observed in Group I, II and III were 22.58, 22.22 and 23.22 per cent respectively. No significant difference could be detected between the three groups. This was in accordance with Carrillo-Gonzalez *et al*. (2019), who found no significant difference in embryo yield after adding L-carnitine in IVM medium before cryopreservation. However, Hochi *et al*. (2001) observed a higher fertilisation rate of 69 per cent following vitrification of bovine oocytes in basic maturation medium, which was higher than that of control group in the present study. The success rate of vitrification depends on many factors, of which vitrification technique, cryodevices and oocytes are the main factors. Factors in technique includes the rate of cooling and warming, viscosity of the medium and volume of vitrification solution (Arav, 2014). During vitrification, the oocytes should be loaded on to cryoloop with a minimal volume (< 2 µL) of vitrification solution within 45 -60 s (Nakayama *et al*., 2020). Even slight over exposure of oocytes in vitrification solution beyond the recommended time may cause depolymerization of tubulin within the oocytes (Aman and Parks, 1994). Damage to the meiotic spindle can change the orientation of chromosomes and thus limit the fertilisation capabilities of oocytes (Eroglu *et al*.,1998). In order to overcome cryopreservation induced zona hardening and premature cortical granule release which can inhibit sperm penetration and fertilisation, many of the researchers adopted ICSI that increased the fertilisation rate.

Conclusion

The survival rate of vitrified bovine oocytes can be enhanced by supplementing niacin than L-carnitine in *in vitro* maturation medium. However further studies are warranted with addition of these agents in vitrification and IVF media to improve the fertilisation rate following vitrification.

Conflict of interest

The authors declare that they have no conflict of interest.

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