Effect of cholesterol loaded methyl-β-cyclodextrin on ovine oocytes during chilling and vitrification

H. ATALLA[1,2]*, K. DEMIR[2], R. ARICI[2], S. YAGCIOGLU[2], A. ESER[2], N. ERSOY[2], N. COSKUN[2], I. ALZUHEIR[1], M. EVECEN[2], S. BIRLER[2], S. PABUCCUOGLU[2]

[1]*Dept. of Veterinary Medicine, An Najah National University, Nablus - PALESTINE*
[2]*Dept. of Reproduction and A.I, Faculty of Veterinary Medicine, Istanbul University, Istanbul - TURKEY*

**Corresponding author: hatematalla@najah.edu - hatematalla@hotmail.com*

ABSTRACT

This study was conducted to evaluate the protective effects of cholesterol pre-incubation on ovine oocytes exposed to cold stress and vitrification. In the first experiment, cumulus oocyte complexes harvested from ovaries of slaughtered ewes were exposed to 2 mg/ml cholesterol loaded methyl-ß-cyclodextrin (CLC) in maturation medium for 2, 4 or 24 h. Oocytes were then exposed to cold stress at 4°C for 30 min, and the viability and chromosome abnormality of oocytes were evaluated. Our results showed no significant differences between treatment groups in regarding to oocyte viability or ooplasm integrity. However, oocytes exposed to cold stress without pre-incubation with CLC had significantly higher chromatin dispersion ($P<0.05$) than pre-incubated oocytes with CLC. In second experiment, oocytes were exposed to 2 mg/ml CLC in maturation medium for 2 h and vitrified after maturation in conventional straws (0.25 ml; CS) or in open pulled straws (OPS). Osmotic stress of vitrification media to oocytes was also evaluated. Oocytes pre-treated with CLC had a significantly higher viability rate and ooplasm integrity than non-treated oocytes after vitrification in OPS ($P < 0.05$). The findings of this study also show that CLC pre-incubation of ovine oocytes reduce the chromosome degeneration after vitrification in both OPS and CS.

Keywords: Ovine, Oocyte, Vitrification, Cholesterol loaded methyl-ß-cyclodextrin

RÉSUMÉ

Effet de la méthyl-β-cyclodextrine chargée de cholestérol sur les ovocytes ovins pendant la réfrigération et la vitrification

Cette étude a été menée pour évaluer les effets protecteurs d'une pré-incubation dans du cholestérol d'ovocytes ovins exposés au froid et à la vitrification. Dans la première expérience, des ovocytes de prélevés sur les ovaires de brebis abattues ont été exposées à 2 mg/ml de méthyl-ßcyclodextrine chargée en cholestérol (CLC) pendant 2, 4 ou 24 h. Les ovocytes étaient alors exposé au stress du froid à 4°C pendant 30 min. La viabilité et les anomalies chromosomiques des ovocytes ont été évalués. Nos résultats n'ont montré aucune différence significative entre groupes sur la viabilité des ovocytes, ou l'intégrité de l'ooplasme. Cependant, les ovocytes exposés au stress du froid sans préincubation avec CLC avait une dispersion de chromatine significativement plus élevée ($P<0,05$) que des ovocytes pré-incubés avec CLC. Dans la deuxième expérience, les ovocytes ont été placés pendant 2 heures dans un milieu contenant 2 mg/ml de CLC et vitrifié après maturation dans 0,25 ml de CS ou OPS. Le stress osmotique du milieu de vitrification sur les ovocytes a également été évalué. Les ovocytes prétraités par CLC présentaient un taux de viabilité et une intégrité de l'ooplasme significativement plus élevés que les ovocytes non traités après vitrification en OPS ($P < 0.05$). Les résultats de cette étude montrent également que la pré-incubation en CLC des ovocytes ovins réduit la dégénérescence chromosomique après vitrification dans les milieux CS et OPS.

Mots-clés : Ovins, Ovocytes, Vitrification, Cholestérol, méthyl-ß-cyclodextrine

Introduction

Female gamete cryopreservation is considered one of the important processes of assisted reproductive technologies for both human and other mammals. This technique is used to cryopreserve genetic resources as well as to preserve human gametes for long period [11]. To date, the sensitivity of the large size cell membrane and the damage of the cell cytoskeleton upon their exposure to cryoprotectants, chilling and freezing are the major challenges for mammalian oocyte cryopreservation [19]. Several achievements and progresses on freezing and thawing of human, mouse, equine and bovine oocytes have been made [6-8, 21]. However, vitrification of small ruminant oocytes, especially ovine oocytes, resulted in the low developmental rates after vitrification of both immature and mature oocytes [4, 15]. To increase the cooling efficiency and cooling rate, different cryodevices were tried. Oocytes were frozen in cryotube [15, 17, 18], cryoloops [17, 18], plastic straws [9, 13], and open pulled straws [13, 20]. Direct chilling injuries, osmotic stress and toxicity of the cryoprotectants mostly affect the plasma cell membrane, especially during the transition from the fluid phase to the gel phase of the cooling process [16]. Moreover, disorganization of the spindle apparatus with chromosomal loss and nuclear fragmentation are major consequences of cryopreservation [10].

Oocyte plasma membrane is the first and main cellular structure affected during cryopreservation and warming processes. Its phospholipid, cholesterol and protein composition play a significant role during cooling, freezing and warming of oocytes. Membranes with high cholesterol concentrations are more resistance to osmotic and thermal stress during cryopreservation [6]. Membrane cholesterol:

phospholipid ratio is a crucial determinant of the fluidity and stability of a membrane during cryopreservation. Low ratios such as those in boar and stallion are associated with less successful cryopreservation, while high ratios in human and bull result in more successful cryopreservation of male gametes [14]. To increase membrane fluidity and permeability at low temperatures, cholesterol can be added to the plasma membrane to provide an alternative method for stability of oocyte during cryopreservation [16]. Recently cyclodextrins were used as a carrier molecule to incorporate cholesterol into plasma membranes of male and female gametes. In previous studies using cholesterol-loaded-β-cyclodextrin (CLC) to modify the cell membranes of rabbit and bovine oocytes, cryopreservation viability is enhanced successfully [6, 12, 14, 16, 19]. Therefore, the objectives of this study were to determine (1) if the cryosurvival of in vitro matured ovine oocytes could be improved by the treatment of oocytes with CLC prior to chilling or vitrification, and (2) if the types of cryodevices could affect the cryosurvival.

Materials and Methods

All chemicals were obtained from Sigma-Aldrich Company (St. Louis, MO, USA) unless otherwise mentioned.

RECOVERY AND MATURATION OF CUMULUS-OOCYTE COMPLEXES

Cumulus oocyte complexes (COCs) were obtained and matured *in vitro* as reported previously [3]. Briefly, sheep ovaries obtained from a local slaughterhouse were brought to laboratory in Dulbecco's phosphate buffer saline (PBS) supplemented with penicillin G (250 IU/ml), streptomycin sulfate (125 µg/ml) and neomycin (125 µg/ml) at 30-35°C. COCs were recovered by slicing the ovaries with scalpel blade. After washing three times, COCs (groups of 35-40) were transferred into a 600 µl of TCM-199 maturation medium supplemented with 10% FCS, 1 mM Na-pyruvate, 10 µg/ml FSH and LH 10 µg/ml under mineral oil and incubated for 22 to 24 h at 38.5°C in a humidified atmosphere of 5% CO_2.

Matured COCs were denuded by vortexing in HSOF medium containing 0.2% (w/v) hyaluronidase for 90 seconds. Viability of oocytes and morphological abnormalities of both zona pellucida and vitellus were evaluated by staining with 0.1% trypan blue (TB). Hoechst stain was used to evaluate chromosome abnormalities of oocytes.

PREPARATION OF CHOLESTEROL-LOADED METHYL-CYCLODEXTRIN (CLC)

The CLC was prepared as described by Purdy and Graham [12]. Briefly, 200 mg cholesterol was dissolved in 1 mL of chloroform. In a separate glass tube, 1 g of methyl-β-cyclodextrin was dissolved in 2 mL of methanol. A 0.45 mL aliquot of the cholesterol solution was added to the cyclodextrin solution and stirred until the combined solution was clear. The mixture was then poured into a glass petri dish and the solvent was removed using a stream of nitrogen gas. The resulting crystals were allowed to dry for an additional 24 h and aliquoted in a glass container at 22°C. A working solution of the CLC was prepared by adding 50 mg of CLC to 1 mL of synthetic oviduct fluid medium supplemented with Hepes (HSOF) at 37°C and mixing the solution briefly by vortex prior to use.

TRYPAN BLUE STAINING FOR VIABILITY OF OOCYTES

Trypan blue was prepared by dissolving TB in PBS (pH=7.0) with a concentration of 0.1%. Denuded oocytes were placed in 50 µl droplet of TB solution for 10 minutes at room temperature, washed with PBS to remove the residual stain and observed under the stereomicroscope (x40). Oocytes stained with blue color were recorded as non-viable (dead), while oocytes that have not incorporate the stain were considered viable (live) (Figure 1). Oocytes with defective membrane or shrinkage cytoplasm were recorded as abnormal [1, 5].

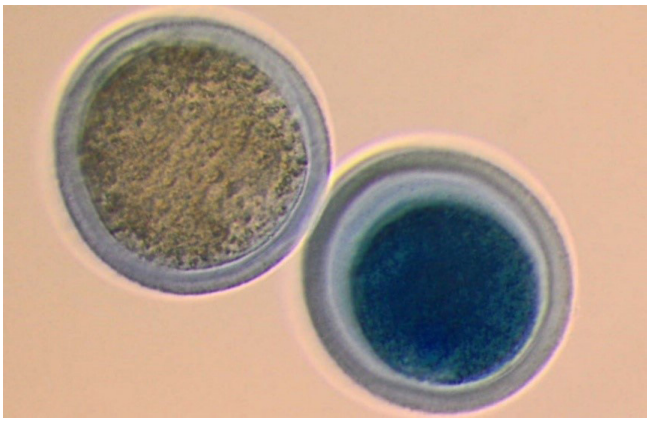

Figure 1: Live oocyte (on the left) and dead oocyte (on the right).

HOECHST STAINING FOR CHROMOSOMAL DEGENERATION OF OOCYTES

For evaluation of chromosomal degeneration; oocytes were incubated with 5 µg/ml Hoechst prepared in HSOF medium for 20 minutes at darkness. After incubation, a group of 10-15 oocytes was transferred to 20 µl of mounting medium on a cover slide with four corners were pre-waxed for slide adhesion and evaluated under the fluorescence microscope (x400). Oocytes that had any chromosomal aberrations, fragmented chromosomes, diffuse or undefined chromatin were considered degenerated (Figure 2 et 3) [9, 16].

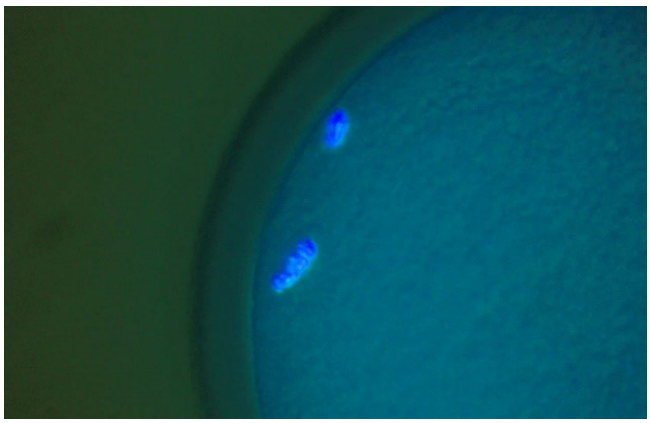

Figure 2: Oocyte with intact chromosomes.

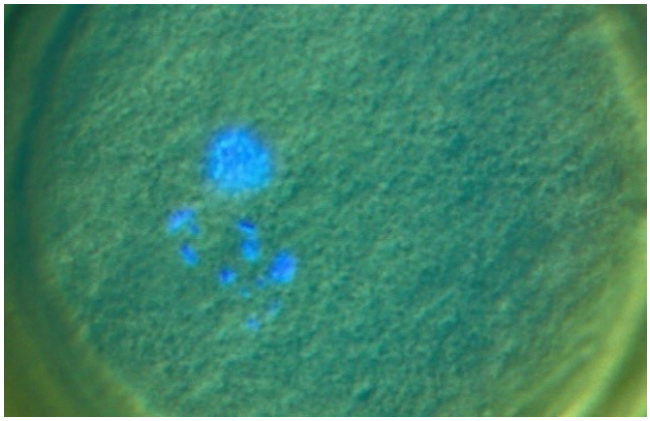

Figure 3: Oocyte with degenerated chromosomes.

Vitrification and warming

Vitrification solutions used with slight modifications in this study were as previously described by Shirazi et al. [15] and Sprícigo et al. [16]. A basic medium (BM) consisted with HEPES-buffered TCM-199 was supplemented with 20% FCS. An equilibration medium (VS1) prepared by dissolving 1 ml of ethylene glycol (10% EG) and 1 ml dimethylsulfoxide (10% DMSO) added to 8 ml of BM. The final vitrification solution (VS2) composed of 20% EG, 20% DMSO and 0.5 M sucrose dissolved in BM.

Denuded oocytes were exposed to BM for 1 min, VS1 for 3 min and to VS2 for 30-45 sec, respectively. Afterward, oocytes were loaded in sets of 5-6 into straws or OPSs followed by direct immersing into liquid nitrogen [20]. The packaging of oocytes for straws and OPS was performed according to Mahmoud et al. [9] and Vajta et al. [20], respectively.

Warming was performed by emptying the straws after 30 sec. in a warm water bath (37°C). OPSs directly pulled out into empty dishes by their capillarity. Oocytes were transferred into 1 M sucrose prepared in BM, incubated for 3 min at 38.5°C, and then put consecutively into 0.5 M and 0.25 M sucrose for 30 sec each.

EXPERIMENTAL DESIGN

Experiment I: cold stress

To investigate the effects of the incubation with cholesterol-loaded methyl-β cyclodextrin (CLC) on the tolerance to cold stress, ovine oocytes were exposed to cold stress (4°C for 30 min) after *in vitro* maturation and exposure to CLC in different time periods. Cumulus-oocyte complexes were distributed into five groups as follows; COCs in negative control group (G1) were matured without CLC and evaluated without submission to cold stress. Oocytes in positive control group (G2) were matured without CLC and evaluated after cold stress. For three time periods used in this study, COCs were incubated with 2 mg/ml CLC for the last 2 h (G3), 4 h (G4) or the whole maturation period 24 h (G5); and were exposed to cold stress. Oocytes were then transferred to HSOF medium and stained with trypan blue (TB) for viability and ooplasm integrity and stained with Hoechst 33342 to investigate chromosomal damage.

Experiment II: osmotic stress and vitrification

Based on the results of Experiment I, it was aimed to investigate the effects of the 2 h incubation of oocytes with 2 mg/ml CLC on the tolerance to osmotic stress of vitrification/warming media and vitrification by using straws or open pulled straws. The osmotic stress was determined after oocytes' exposure to vitrification and warming media without immersion into liquid nitrogen.

Cumulus oocyte complexes were assigned randomly into four groups; two groups were incubated with 2 mg/ml CLC for 2 h, one exposed to vitrification media (CLC+/VIT+) and the other left as control (CLC+/VIT-). The effect of vitrification media was also tested by incubation without CLC and with exposure to vitrification media (CLC-/VIT+) or without exposure to vitrification media (CLC-/VIT-).

To study the effects of CLC in vitrification, COCs were assigned randomly into 4 groups; matured without using CLC and then vitrified in conventional 0.25 ml straw (CLC-/S) or in open pulled straws (OPS) (CLC-/O). In the other two groups, oocytes were matured in the presence of 2 mg/ml CLC and then vitrified in conventional 0.25 ml straw (CLC+/S) or in OPS (CLC+/O). All vitrified oocytes were stored in liquid nitrogen. After 2-4 weeks, oocytes were warmed and stained with TB to investigate their viability and ooplasm integrity or with Hoechst for their nuclear status.

STATISTICAL ANALYSIS

The data were analyzed using IBM SPSS Version 20. Statistics of the data were performed using chi-square test. P-values less than 0.05 were considered to be statistically significant.

Results

Pre-incubation effects of ovine oocytes to CLC showed in Table 1. No statistically significant differences in regarding to viability were found between the different CLC incubation periods compared to negative or positive control groups. As shown in Table 1; 2 h, 4 h and 24 h incubation of oocytes with CLC decreased the chromatin dispersion significantly (P<0.05) after exposure to cold stress (8.1 to 10.1% vs 19.7% in control).

The viability rates of oocytes pre-incubated for 2h with CLC and exposed to vitrification and warming solutions showed no significant differences compared to viability rates of oocytes not incubated with CLC (95.2% vs 91.5% respectively, P>0.05) (Table 2). Although chromosomal dispersion rate of CLC-free matured oocytes was higher than oocytes pre-incubated with CLC (32.7% and 19.3%, respectively), the difference was not significant statistically (p>0.05).

Table 3 shows the viability and chromosome integrity of oocytes pre-incubated for 2h with CLC and suspected to vitrification by using different methods. Oocytes pre-incubated with CLC and vitrified by OPS method had significantly higher viability and lower chromatin dispersion rates (63.8% and 21.4%), than non CLC pre-incubated OPS vitrified oocytes (44.9% and 36.5%) respectively (P<0.05). In contrast, pre-incubation with CLC had no effects on

Groups		Oocytes stained with Trypan Blue		Oocytes stained with Hoechst	
CLC	Cold Stress	N	Viable (%)	N	Degenerated Chromosome (%)
No	No	156	154 (98.7)	116	4 (3.4)[a]
No	Yes	147	143 (97.3)	137	27 (19.7)[c]
2 h	Yes	143	141 (98.6)	136	11 (8.1)[ab]
4 h	Yes	149	144 (96.6)	142	12 (8.5)[ab]
24 h	Yes	149	146 (98.0)	129	13 (10.1)[b]

[abc] values in a column with different superscript differ (P <0.05).
Data are cumulative values of 4 replicates for each group.

Table I: Viability and chromosome degeneration of oocytes after cold stress

Groups		Oocytes stained with Trypan Blue		Oocytes stained with Hoechst	
CLC	Vitrification	N	Viable (%)	N	Degenerated Chromosome (%)
No	No	59	58 (98.3)[ab]	53	11 (20.8)
No	Yes	59	54 (91.5)[b]	55	18 (32.7)
Yes	No	61	61 (100.0)[a]	58	15 (25.9)
Yes	Yes	63	60 (95.2)[ab]	57	11 (19.3)

[ab] values in a column with different superscript differ (P <0.05).
Data are cumulative values of 5 replicates for each group.

Table II: Effects of osmotic stress of vitrification and warming solutions on viability and chromosome degeneration of oocytes

Groups		Oocytes stained with Trypan Blue		Oocytes stained with Hoechst		
CLC	Vitrification method	N	Viable (%)	N	Metaphase II (%)	Degenerated chromosome (%)
No	Straw	93	57 (61.3)[a]	81	39 (48.14)[ac]	25 (30.9)[ab]
No	OPS	89	40 (44.9)[b]	74	25 (33.78)[a]	27 (36.5)[a]
Yes	Straw	110	61 (55.5)[ab]	98	64 (65.30)[bd]	15 (15.3)[c]
Yes	OPS	116	74 (63.8)[a]	98	57 (58.16)[cd]	21 (21.4)[bc]

[abc] values in a column with different superscript differ (P<0.05).
Data are cumulative values of 7 replicates for each group.

Table III: Effect of vitrification by straw or OPS on viability and chromosome degeneration of oocytes

oocyte viability rates when cryopreserved in straws without pre-incubation with CLC (61.3% vs 55.5%). The CLC pre-incubation resulted in lower chromatin dispersion rate than non incubation in straw group (15.3% vs. 30.9%, $P<0.05$).

Discussion

In this study, it has been studied the protectant effect of cholesterol loaded methyl-ß-cyclodextrin on viability and chromosome status of ovine oocytes matured *in vitro* after exposing to cold stress and vitrification. Oocyte plasma membrane considered the most affected part during cryopreservation and warming processes. Affected plasma membrane may lead to further damage of cytoplasmic and nuclear organelles [6]. Chromosomal damage caused by cryopreservation considered one of the major reasons that impair the ability of oocyte to be fertilized and developed to the blastocyst stage [17]. Phospholipid composition of oocyte plasma membrane, particularly cholesterol: phospholipids ratio determines its sensitivity to cooling. Membranes with a high ratio of cholesterol could be more resistant to cooling and freezing [14, 16]. The lipid content of ovine oocyte is lower than that of bovine oocyte which decreases embryo quality and developmental rates [4, 15]. Enhancement of the membrane cholesterol composition of immature ovine oocytes in this study, had no effect on the oocyte viability, however, chromosome degeneration was significantly decreased after cold shock. This supports the suggestion of protectant effect of CLC which can indirectly protect the nuclear material during cooling [16].

Non-CLC pretreated oocytes vitrified by the straw method have a significantly higher survival rates compared to ones vitrified by OPS method. These findings are in agreement with the study of Arcarons et al. [2] which showed that the straw is a more efficient carrier for vitrification than OPS for the cryopreservation of bovine oocytes. On the contrast our results were in disagreement with the findings of Quan et al [13] in which 59.1%, of bovine oocytes verified with OPS reached MII phase comparing to 34.9% verified by conventional straw method.

The CLC pre-incubation caused no significant increase in oocyte viability after straw vitrification but chromosome integrity rates were highly significant in this group. On the other hand, the effects of CLC pre-incubation significantly increased oocyte viability and chromosome integrity of oocytes vitrified in OPS after incubation with CLC.

It was reported that cholesterol supplementation prior to vitrification has a positive effect on oocyte morphology, and viability of bovine oocytes [6, 16]. Previous study demonstrated that cleavage and eight-cell rates were higher in CLC-treated bovine oocytes. In contrast, Trigos et al. [19] reported that treatment with CLC increased cholesterol in rabbit oocytes, but did not improve developmental competence of cryopreserved oocytes. Oocytes vitrified by straws and OPS after treatment with CLC were higher in maturity rates (65.30% and 58.16%, respectively) than oocytes not treated with CLC (48.14% and 33.78%, respectively). Our findings report for the first time the positive effect of CLC pre-incubation on the ovine oocytes survival and development rates. Pre-incubation with CLC in vitrification media might enhances the incorporation of cholesterol into ovine oocytes membrane or cytoplasm before vitrification and improve oocytes cryoresistance. In addition, CLC pre-incubation decrease chromosome dispersion rates after the two vitrification methods in comparison to non CLC pretreated oocytes. These results indicated that CLC could protect chromosome integrity by protecting oocyte membrane against freezing shock when oocytes vitrified by both freezing devices (straw and OPS). In other words, the presence of CLC during the pre-vitrification stage could provide protection to the membrane of oocytes during vitrification by OPS but had no significant effects on oocytes vitrified by straws. These results indicated that CLC could protect chromosome integrity by protecting oocyte membrane against freezing shock when oocytes vitrified by OPS. It is not clear if CLC can inter to oocyte cytoplasm or not; so, we can say that CLC indirectly could affect and protect cytoplasm and nuclear material by its effects on oocyte membranes. According to the results, it's obvious that the increased maturation rates of CLC groups were the result of decreased chromosome dispersion rates of these groups.

In conclusion, although, the results presented in this study display that CLC improved ovine viability after vitrification and warming especially when oocytes were vitrified by conventional straws, simultaneously CLC could improve chromosome integrity of oocytes vitrified with OPS. More studies are required to emphasis the mechanism of oocyte protection and to increase oocytes nuclear integrity by using cytoskeleton protecting particles beside CLC.

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