



One / Two Days Preservation of goat oocytes at refrigerator and their maturation *in vitro*

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Abstract

This study was conducted to evaluate the feasibility of one / two days preserving immature goat oocytes without freezing. Cumulus oocyte complexes (COCs) were obtained from ovaries of slaughtered goats. Selected COCs were kept in tissue culture medium (TCM-199) and 10% Fetal Calf Serum (FCS) and stored at 4°C for 1 or 2 days. After preservation, oocyte morphology and viability were evaluated. Post-warmed stored and Non-stored oocytes were cultured in TCM-199 and 10% FCS in CO₂ incubator for 30 h. Oocyte maturation was indicated by cumulus expansion after *in vitro* culture. Data analysis revealed a high percentage of morphologically normal and viable oocytes after preservation either for 1 or 2 days (70, 60 vs. 60, 50%, respectively) with low percentage of damaged oocytes. There was no significant difference in the maturation of stored oocytes for 1 or 2 days in comparison to fresh oocytes although the value of the fresh oocytes was higher (55, 50 vs. 65%, respectively). Thus, we have developed a simple method for preserving immature goat oocytes by refrigeration at 4°C for two days without evident damage of oocyte and keeping oocyte developmental competence.

Keywords: preservation, goat, oocytes, *in vitro* maturation

Introduction

Oocyte preservation and transport of oocytes are important aspects of research and experimentation which oftentimes needs to be performed at a convenient time or place that is different from the sites of ovary and oocyte collection. Maintaining meiotic arrest during preservation is critically important to improve the quality of *in vitro* matured oocytes. At the present time, slow freezing and vitrification

are the only practical methods for preservation of oocytes (Abd-Allah, 2003). However, slow-freezing method proved that mammalian oocytes were highly sensitive to freezing and the formation of ice crystals. Also, it may negatively affect subsequent nuclear and cytoplasmic reorganization of the germinal vesicle stage oocytes and even damage mitochondria and microtubules including those comprising the meiotic spindle (Nagashima *et al.*, 1994). In addition, cryoprotectant toxicity and osmotic injury to the oocytes often occur during the thawing/warming phase (Zhou and Li, 2009). These problems hampered the application of oocyte cryopreservation.

The effects of preservation conditions on maturation of oocytes were found to depend on the animal species. An early report showed that porcine oocytes still displayed maturation capability and developmental capacity after 24 h preservation at 20°C in TCM-199 (Jiang, 1992). Also, Wakayama *et al.* (2004) demonstrated that at least a few percent of mouse oocytes stored at room temperature retained the potential for full-term development, but irreversible injuries not only damage the cytoplasm but also the spindle apparatus. Likewise, Kim *et al.* (2006) reported that mouse oocytes could be preserved at room temperature and parthenogenesis developmental competence was not affected by exposure of oocytes to room temperature for 1, 2 or 4 h in Dulbecco's Phosphate Buffered Saline.

Trypan blue stain has been used previously to detect oocyte viability (Gupta *et al.*, 2002 and Abd-Allah *et al.*, 2008) and it is based on plasma membrane integrity (Schrek, 1936). Dead oocytes displayed a dark blue ooplasm with translucent cumulus cells. Moreover, it has been reported that the trypan blue exclusive test is a useful and rapid method to assess the initial quality and viability of oocytes (Gupta *et al.*, 2002 and Abd-Allah *et al.*, 2008).

The objective of this study was to determine how long could the goat oocytes be preserved in the TCM-199 and 10% FCS without freezing at refrigerator.

Material and Methods

Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated.

Collection of Oocytes

Slaughtered goat ovaries were collected and transported within 3 h to the laboratory in sterilized saline containing 100 IU/ml penicillin and 0.05 mg/ml streptomycin and maintained at 37°C.

Cumulus oocyte complexes (COCs) were obtained by aspiration of 2-6 mm follicles from ovaries as described previously (Abd-Allah, 2010).

The COCs were examined under a stereomicroscope and only those with more than three complete layers of cumulus cells and a fine granulated homogenous ooplasm were used.

Preservation of goat oocytes at 4°C

COCs were equilibrated at room temperature in 20 in droplets of 100 µl of TCM-199+10% FCS covered by mineral oil at 4°C and then stored at 4°C in refrigerator for 1 or 2 days.

Warming of Stored oocytes

Petri-dishes contained stored oocytes were removed from refrigerator and held at room temperature for 2 minutes and then washed 2-3 times in a culture medium (TCM-199 + 10% FCS) and co-cultured in 50 µl drops of this medium at 38°C in a humidified atmosphere of 5% CO₂.



Fig. 1: Morphological Normal and Abnormal of Post-warmed Stored Oocytes

A: Abnormal post-warmed stored oocytes (Rupture of zona pellucid).

B: Normal post-warmed stored oocytes.

Assessment of survival of oocytes

Morphologically Assessment

The post-warming survival of oocytes was observed under a stereomicroscope. Oocytes were judged morphologically as survivors when the spherical

and symmetrical shape had no signs of lysis, membrane damage, degeneration or leakage of the cellular content (Fig. 1); Oocytes were considered abnormal when a ruptured zona pellucid or fragmented cytoplasm with signs of degeneration were present (Dhali *et al.*, 2000) (Fig. 1).

Examination of oocyte viability using the trypan blue exclusion test

The trypan blue exclusion test was used to provide an assessment of cell membrane integrity.

Trypan blue solutions (0.05%) were prepared by dissolving trypan blue in phosphate buffer saline (PBS) (pH = 7.0) and the staining of oocytes was performed at room temperature (Gupta *et al.*, 2002 and Abd-Allah *et al.*, 2008).

All post-warmed stored oocyte were examined for viability using the trypan blue exclusion test. Immature oocytes were categorized on the basis of the degree of dye exclusion. Unstained oocytes were classified as viable and fully stained oocytes as dead (Gupta *et al.*, 2002 and Abd-Allah *et al.*, 2008).

In vitro maturation of stored Oocytes at 4°C

Morphologically normal postwarmed COCs were washed twice with TCM-19 supplemented with 10% FCS and 50 µg/ml gentamycin sulphate.

Maturation was performed in 50 µl drops (10 COCs/drop) of the same medium used for washing and was supplemented with 10 µl/ml pregnant mare serum gonadotropin (PMSG) (Folligon, Intervet, Cairo), 10 µl/ml human chorionic gonadotropin (hCG, Pregnyl, Nile Company for Pharmaceuticals and Chemical Industries, Cairo). The reaction with the postwarmed COCs was conducted with mineral oil and incubated for 48 h at 38.5°C and in an atmosphere of 5% CO₂ to evaluate the maturation. The oocytes showed cumulus expansion were considered to be completely matured. Non-Stored oocytes subjected to the same *in vitro* maturation technique were used as controls.

Results and Discussion

The results of the present study (Table 1) revealed a percentage of post-warming morphologically normal and viable immature oocytes stored at 4°C in refrigerator for 1 or 2 days.

The differences between the percentages of morphological normal oocytes stored at one day and two days procedure were non-significant ($p < 0.05$), although the value of the one day stored oocytes was higher (70, 60 vs. 60, 50 %, respectively).

Table 1. Frequency of different observations of stored oocytes for 1 or 2 days.

Criteria	Stored Oocytes at 4°C	
	1Day	2 Days
Preservation periods (Day)	1Day	2 Days
No. of Oocytes Examined	100	100
Postwarmed Normal oocytes (%)	70 ^a	60 ^a
Damage oocytes (%)	30 ^a	40 ^a
Morphological Observation (%)		
Ruptured zona pellucida	50	60
Shrinkage of cytoplasm	30	25
Leakage of cell content	20	15
Viability of Oocytes (%)		
Trypan blue Exclusion test	60 ^a	50 ^a

Within the same raw, Values with the same superscript are insignificantly different at $P < 0.05$.

In the present study, the stored oocytes were preserved at 4°C for one or two days, it was observed that there was no significant change recorded in the viability of oocytes among both days. (60% vs.50 %, respectively).

Table 2. Developmental competence of fresh and Stored oocytes.

Criteria	Fresh Oocytes	Stored Oocytes	
		1	2
Preservation periods (Day)	-	1	2
Number of Cultured Oocytes	100	60	50
Matured Oocytes (%)	65(65%) ^a	33(55%) ^a	25(50%) ^a
Cumulus Expansion of matured oocytes (%)			
CE+	40(40%) ^a	22(36.7%) ^a	17(34%) ^a
CE+/-	25(25%) ^a	11(18.3%) ^a	8(16%) ^a
CE-	35(35%) ^a	27(45%) ^a	25(50%) ^a

Within the same raw, Values with the same superscript are insignificantly different from each other ($P < 0.05$).

CE+ : Cumulus-full expanded oocytes.

CE+/- : Cumulus-partially expanded oocytes (whenever there were few expansion of cumulus layers or cumulus cells were non-homogeneously spread and clustered cells were still observed or moderate expansion of cumulus layers)

CE- : Cumulus-non expanded oocytes.

Our statistical analysis (Table 2) showed a non-significant difference ($p < 0.05$) in the maturation rate of stored oocytes either for 1 or 2 days in comparison to those of fresh oocytes although the value of the fresh oocytes was higher (55, 50 vs. 65%, respectively).

Strikingly, when COCs were stored at 4°C for 2 days in TCM-199+10% FCS, more than 60% oocytes were still morphologically normal and more than 50% oocytes were matured at 30 h (Tables 1, 2).

It is generally accepted that goat oocyte preservation is highly significant for agriculture and biomedicine. It also has implications for human assisted reproduction technologies. However, many problems still exist concerning conventional goat oocyte preservation methods, such as low temperature sensitivity, cryoinjuries, toxicity of preservation solution and low success rates. Therefore, it is important to determine new methods for short-term oocyte preservation and long distance transportation, which not only avoids toxicity and cryoinjuries, but also allows synchronous maturation. Here, we report that 4°C is the optimal temperature for preservation of oocytes in TCM-199 + 10% FCS. After preservation for 2 days, oocytes are still viable, remain at the germinal vesicle stage, and have undergone beneficial cytoplasmic changes.

The present results demonstrated that although goat oocytes blocked with Protocol 4°C for 2 days retained the same competence of development as non-treated oocytes.

Conclusion

It is concluded that, due to the high performance obtained, the preservation of goat oocytes at refrigerator used in this study is considered more suitable for the short term preservation of goat oocytes.

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