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Effects of storage conditions of dromedary camel ovaries on the morphology, viability and development of antral follicular oocytes

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Abstract

The purpose of this study was to evaluate the effect of cooling ovarian tissue on the morphology, viability and developmental ability of camel antral follicular oocytes. Ovaries were maintained in saline solution (0.9%) at 4 or 20°C for 6, 12, 18 or 24 h. After storage, COCs were recovered from dromedary ovaries and were evaluated for morphology and viability by trypan blue exclusion test. To assess the viability of stored oocytes, morphologically normal and live COCs isolated from ovaries stored at 4°C for 24 h and at 20°C for 6 h were *in vitro* matured (IVM) in TCM 199 + 10 µg/ml FSH + 10 IU hCG/ml + 10% FCS + 50 µg/ml gentamycin for 30 h at 38.5°C under 5% CO_2 and 95% humidity. The percentage of morphologically normal and live oocytes was significantly reduced in ovarian tissue stored at 20°C for 12, 18 or 24 h. Follicular oocytes stored in 4°C for up to 24 h or 20°C for 6 h presented the same growth pattern as fresh follicular oocytes. We conclude that storage of camel ovaries at 4°C for up to 24 h or at 20°C for up to 6 h did not affect the morphology and viability of oocytes or their ability to mature *in vitro*.

Keyword: camel, development, oocytes, storage conditions, viability.

Introduction

The ovarian follicle is the basic structural and functional unit of the mammalian ovary that provides the microenvironment necessary for oocyte growth and maturation. More than 90% of oocytes within an ovary are enclosed in pre-antral follicles (Saumande, 1991). In the last decades, many studies have been carried out focusing on this population of ovarian follicles. Various methods have been developed to isolate and culture pre-antral follicles from cow (Gutierrez *et al.*, 2000), buffalo (Abd-Allah, 2009) and camel (Abd-Allah *et al*., 2008) ovaries. Furthermore, recently much attention has been given to the short-term preservation at low temperatures (Silva *et al.*, 2000; Lucci *et al.*, 2004) and cryopreservation (Candy *et al.,* 1997; Abd-Allah, 2009) of pre-antral follicles from several mammalian species.

The short-term preservation is especially important for ovary transportation, mainly in the case of farm or endangered animals, when the ovarian donor is far away from specialized laboratories. Techniques for short-term storage of ovaries were already developed for goats (Silva *et al*., 2000) and cows (Lucci *et al.*, 2004). In these studies, the temperatures of 4 and 20°C were tested for the preservation of pre-antral follicles. Despite differences within species, results were similar. In general, the most suitable temperature was 4°C, allowing preservation of antral follicles for periods as long as 18 or 24 h, while 20°C was able to preserve the follicles for only 6 h.

In camel, no information is available concerning the short-term storage of antral follicles within the ovaries at low temperatures. However, it is well known that camel oocytes are sensitive to chilling, especially under 15°C. This cooling sensitivity is attributed to the high lipid content of camel oocytes (Nili *et al*., 2004), and it is clear that some form of tolerance to low temperatures is gained when their lipid content is reduced. Nevertheless, these studies were performed with oocytes from antral follicles, and it is known that oocytes within antral follicles differ from fully grown oocytes in several structural and functional aspects (Shaw *et al*., 2000). These differences make antral follicle oocytes less susceptible to damage caused by hypothermic conditions (Gosden, 2000), and one of the differences is that a smaller amount of cold-sensitive intracytoplasmic lipids are found in these oocytes (Shaw *et al*., 2000). If camel antral follicles can be successfully preserved at lower temperatures, as in other species, the use of these follicles would be a reliable alternative for the preservation of camel oocytes.

Trypan blue stain has been used previously to detect oocyte viability. Dead oocytes displayed a dark blue ooplasm with translucent cumulus cells. Moreover, it has been reported that Trypan blue stain is a useful and quick method to assess the initial quality and viability of follicles (Jewgenow and Goritz, 1995; Abd-Allah *et al*., 2008).

Dromedary camel oocytes have been matured *in vitro* for 30 h (Abd-Allah *et al*., 2008) or 36 h (Torner *et al.*, 2003). However, in the present study, we used 30 h for maturation as we reported previously that the best time of maturation of camel oocytes was 30 h (Abd-Allah *et al*., 2008).

The present study aimed to evaluate the effect of storing antral follicles within camel ovaries under

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different temperatures, and to test the ability of these follicles to grow *in vitro* after being stored at 4 and 20°C.

Materials and Methods

All reagents were obtained from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise specified.

Experimental protocol

Storing of ovaries

Ovaries (n = 300) from non-pregnant female dromedary camel were collected at the moment of slaughter at a local abattoir in Cairo, stripped of fat tissue and ligaments, and rinsed in saline solution (37°C).

Collected ovaries (n = 300) were stored at 4 or 20°C for 0, 6, 12, 18 and 24 h (30 ovaries stored for 4°C and 30 ovaries stored for 20°C for each time) in a refrigerator (4°C) or an electric thermo flask (20°C) prior to oocyte collection. Stored ovaries (n = 60) at 4 or 20°C for 0 h were considered as not stored and treated as control fresh group. Temperatures were measured at the beginning of the experiment. Each treatment was repeated 5 times.

Survival of oocytes after storing at 4 or 20°C for 0, 6, 12, 18 and 24 h

Morphological evaluation of follicular oocytes

Dromedary cumulus-oocytes complexes (COCs) were recovered by manual aspiration of follicles at 2–8 mm diameter by using a 20-gauge needle attached to a 10 ml sterile disposable syringe (Abd-Allah *et al.*, 2008).

Released immature camel oocytes were scored for granulose-oocyte cell adhesion as previously described (Combelles and Albertini, 2003): C^{+} for granulosa-enclosed oocytes, $C^{+/-}$ for partially granulose-enclosed oocytes, (whenever there were granulosa cell-free regions on the oocyte surface) and C^{-} for granulosa-free oocytes.

Recovered oocytes were observed under a stereomicroscope (M6C ‑ 10, N9116734, Russia). Oocytes were morphologically classified as normal oocytes if they were spherical, had a symmetrical shape and no signs of lysis, membrane damage, swelling, degeneration or leakage of the cellular content were observed, or as abnormal oocytes if they had ruptured zona pellucida or fragmented cytoplasm with signs of degeneration.

Viability evaluation of follicular oocytes using the trypan blue exclusion test

Trypan blue solutions (0.05%) were prepared by dissolving trypan blue in phosphate buffer saline (PBS; pH = 7.0). Oocyte staining was performed at room temperature for 2 min (Abd-Allah *et al*., 2008).

Trypan blue stain has been used previously to detect oocyte viability. Dead oocytes displayed a dark blue ooplasm with translucent cumulus cells. Moreover, it has been reported that Trypan blue stain is a useful and quick method to assess the initial quality and viability of follicles (Jewgenow and Goritz, 1995; Abd-Allah *et al*., 2008)

Immature oocytes were isolated from antral follicles in Dulbecco PBS (DPBS), washed in culture media (TCM-199 and 10% FCS) and a final cell suspension of 1 ml of culture media was used for the trypan blue exclusion test (Freshney, 2000). The exclusion test provides an assessment of cell membrane integrity as those cells with damaged or non-intact cell membranes permit the passage of the trypan blue toward the nucleus of oocyte.

All oocyte classes of both methods 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 live and fully stained oocytes as dead. Abd-Allah *et al*. (2008) reported that the developmental ability of camel oocytes was not affected by trypan blue staining.

Developmental competence of follicular oocytes

To evaluate the ability of recovered oocytes from stored ovaries on the development *in vitro*, Only recovered oocytes classes C^{+} / $C^{+/-}$ collected from stored ovaries after washing 3 times in washing media to remove the trypan blue stain, were placed in 50 µl drops (10 COCs/drop) of the same medium used for washing, and 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) under mineral oil, at 38.5°C in a 5% CO_2 and 95% humidity for 30 h (Abd-Allah *et al*. (2008). To assess oocyte maturation, cultured COCs were examined for cumulus expansion and extrusion of the first polar body.

Statistical analysis

Data were analyzed using Chi-square analysis (Snedecor and Cochran, 1980).

Results

Morphological and viability evaluation of follicular oocytes

The number of ovaries stored in each temperature was 30 ovaries and the recovery rate of oocytes ranged from 2.5 - 2.7/ovary.

Percentages of oocytes classified morphologically as normal and live after trypan blue test and recovered from ovaries stored at 4 or 20ºC for different periods of time are presented in Table 1.

The temperature of 20ºC proved to be inferior to 4ºC in recovering of granulosa – enclosed oocytes (C^+) derived from antral follicles when ovaries were stored for 12, 18, and 24 h and resulted in significantly reduced percentages of recovered granulosa – enclosed oocytes.

As shown in Table 1, 72-92 and 64-86% of the oocytes from stored ovaries at 4ºC and stored ovaries at 20ºC, respectively, presented normal morphology on 0 h and live oocytes (Table 1), and there were no significant differences between both groups for any of the ratios calculated.

Ovarian storage at 20ºC for 12, 18 or 24 h significantly reduced the percentage of morphologically normal and live growing oocytes compared with stored ovaries at 4ºC for 12, 18 and 24 h ($P < 0.05$; Table 1). The percentages of normal and live growing oocytes in ovaries stored at 20ºC for 12 and 18 h was also significantly lower than in ovaries stored at 20ºC for 6 h ($P < 0.05$).

Table 1. Percentages of morphologically normal and live camel oocytes retrieved from ovaries stored at 4 and 20ºC, scored for granulosa-oocyte adhesion.

Time	Storing Temp.	Recovered oocytes†			Morphological observation			Trypan blue exclusion test		
		C^+	$C^{+/-}$	C^-	C^+	$C^{+/-}$	C^-	C^+	$C^{+/-}$	C^-
0 h††	4ºC	64.5% (49/76)	21.1% (16/76)	14.4% (11/76)	91.8% (45/49)	81.2% (13/16)	72.7% (8/11)	85.7% (42/49)	75% (12/16)	63.6% (7/11)
	20ºC	64.5% (49/76)	21.1% (16/76)	14.4% (11/76)	91.8% (45/49)	81.2% (13/16)	72.7% (8/11)	85.7% (42/49)	75% (12/16)	63.6% (7/11)
6 h	4ºC	60% (48/80)	25% (20/80)	15.7% (12/80)	87.5% (42/48)	80% (16/20)	75% (9/12)	83.3% (40/48)	70% (14/20)	58.3% (7/12)
	20ºC	52.5% (42/80)	21.2% (17/80)	26.2% (21/80)	83.3% (35/42)	70.5% (12/17)	80.9% (17/21)	80.9.% (34/42)	64.7% (11/17)	61.9% (13/21)
12 h	4ºC	60%^{a} (48/80)	25%^{a} (20/80)	15.7%^{a} (12/80)	87.5%^{a} (42/48)	80%^{a} (16/20)	75%^{a} (9/12)	87.5%^{a} (42/48)	80%^{a} (16/20)	75%^{a} (9/12)
	20ºC	41.2%^{a} (33/80)	15%^{a} (12/80)	44%^{a} (35/80)	57.1%^{a} (20/33)	50%^{a} (6/12)	45.7%^{a} (16/35)	54.5% ^{a} 18/33)	41.6%^{a} (5/12)	37.1%^{a} (13/35)
18 h	4ºC	60%^{b} (48/80)	25%^{b} (20/80)	15.7%^{b} (12/80)	87.5%^{b} (42/48)	80%^{b} (16/20)	75%^{b} (9/12)	87.5%^{b} (42/48)	80%^{b} (16/20)	75%^{b} (9/12)
	20ºC	25%^{b} (20/80)	12.5%^{b} (10/80)	62.5%^{b} (50/80)	40%^{b} (8/20/)	30%^{b} (3/10)	24%^{b} (12/50)	35%^{b} (7/20/)	20%^{b} (2/10)	16%^{b} (8/50)
24 h	4ºC	58.9%^{c} (46/78)	23.0%^{c} (18/78)	17.9%^{c} (14/78)	84.4^c (38/46)	77.7%^{c} (14/18)	64.2%^{c} (9/14)	84.4^c (38/46)	77.7%^{c} (14/18)	64.2%^{c} (9/14)
	20ºC	10.2%^{c} (8/78)	5.1%^{c} (4/78)	84.6%^{c} (66/78)	25%^{c} (2/8)	25%^{c} (1/4)	21.1%^{c} (14/66)	12%^{c} (18/33)	0.0%^{c} (0/4)	9%^{c} (6/66)

Values with the same superscript in the same column were significantly different at $P < 0.05$.
C^+ granulosa-enclosed oocytes.
$C^{+/-}$ partially granulosa-enclosed oocytes (whenever there were granulosa cell-free regions on the oocyte surface).
C^- granulosa-free oocytes.
†30 ovaries were stored in each temperature and 2.5 - 2.7 oocytes/ovary were recovered.
††Non-stored fresh oocytes.

Developmental competence of follicular oocytes

When antral follicular oocytes isolated from ovaries stored at 4ºC for 24 h and at 20ºC for 6 h were placed into culture, they grew *in vitro* in the same pattern as fresh oocytes did. Maturation rate was 85% for oocytes recovered from fresh follicles, 80% for oocytes recovered from follicles stored at 4ºC for 24 h and 78% oocytes recovered from follicles stored at 20ºC for 6 h. There were no significant differences between three groups of ovaries (Table 2).

Table 2. Percentages of recovered, normal, live immature and matured camel oocytes from non-stored ovaries and stored ovaries at 4ºC for 24 h or 20ºC for 6 h.

Criteria	Recovered oocytes	Normal oocytes	Live oocytes	Maturation rate†
Non-stored ovaries	85.5% [a] (65/76)	89.2% [b] (58/65)	83.0% [c] (54/65)	85% [d] (46/54)
Ovaries stored at 4ºC for 24 h	82.0% [a] (64/78)	81.2% [b] (52/64)	81.2% [c] (52 /64)	80.0% [d] (42/52)
Ovaries stored at 20ºC for 6 h	73.7% [a] (59/80)	79.6% [b] (47/59)	76.2% [c] (45/59)	77.7% [d] (35/45)

Within the same column, values with the same superscript are insignificantly different from each other ($P < 0.05$).
†Maturation rate: number of matured oocytes/number of lived oocytes.

Discussion

The recovery rate of oocytes per ovary ranged from 2.5 - 2.7%, and this low number of recovered oocytes may be attributed to the ovaries were collected from aged, non-pregnant animals during the non-breeding season.

This study shows for the first time that camel antral follicles successfully survived when ovaries were stored at 4°C. The percentage of normal and live growing oocytes was significantly reduced when ovaries were stored at 20°C for 12, 18 or 24 h. This may be attributed to the growing oocytes have already started their development as they present many granulosa cells in mitotic activity. However, the oocyte, although still arrested at the first meiotic division, is actively synthesizing both protein and RNA (Fair *et al*., 1997; Hyttel *et al*., 1997) and the organelles are dividing and maturing (Hyttel *et al.*, 1997). Then, it is obvious that growing follicular oocytes are in need of nutrients and oxygen, and probably for this reason they could not survive more than 6 h stored at 20°C in a solution poor in nutrients and in a closed flask without an appropriate atmosphere. Conversely, the temperature of 4°C probably lowered the cellular metabolism, slowing down the degenerative process in the ovaries (Silva *et al*., 2000).

In fact, it has been demonstrated that the results presented here confirm that camel oocytes enclosed in antral follicles are not sensitive to a low temperature of 4°C.

Morphological assessment of follicular integrity has been largely used to evaluate the effectiveness of the various treatments to which ovarian follicles are submitted to (Silva *et al*., 2000; Lucci *et al*., 2004). However, additional measurements such as follicular development and oocyte maturation may be used as a reliable evaluation of follicles oocyte viability. *In vitro* maturation of camel oocytes has been successfully performed and a high proportion of oocytes matured (86.2%) after being cultured for 30 h (Abd-Allah *et al.*, 2008). Similarly, in our study, a high percentage of oocytes collected from ovaries stored at 4°C for 24 h or at 20°C for 6 h, matured *in vitro* (78 vs. 85%, respectively) and at similar rates compared to fresh oocytes (85%). However, after *in vitro* culture, a variable percentage of oocytes showed signs of degeneration that consisted of being misshapen with a coagulated cytoplasm. It is known that a variable number of oocytes degenerate during *in vitro* culture (Telfer *et al.*, 2000). Therefore, the culture conditions (which are not well established yet) can be partially attributed, to the degeneration rates observed in the present study. Moreover, oocyte degeneration occurs when follicles are cultured *in vitro* (Figueiredo *et al*., 1994).

Another factor that may have positively influenced oocyte survival is that growing oocytes were collected from antral follicles. We suggest that camel oocytes enclosed in follicles are less sensitive to cold temperature. This may be attributed the lower oocyte cytoplasmic droplets content, which are considered to be partially responsible for the oocyte cooling intolerance (Nili *et al*., 2004).

In conclusion, we demonstrated that storing camel ovaries at 4°C for up to 24 h did not affect the percentage of morphologically normal oocytes, while ovarian storage at 20°C for 12, 18 or 24 h reduced the percentage of morphologically normal oocytes. Moreover, oocytes collected from camel ovaries that were stored at 4°C for up to 24 h or at 20°C for up to 6 h maintained their normal morphology and ability to mature *in vitro,* making the transportation of ovaries over long distances a feasible process. The results presented herein suggested that camel oocytes enclosed in antral follicles are less sensitive to low temperatures. The use of antral follicular oocytes can be a good alternative for the preservation of camel germinal cells.

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