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Effects of storing dromedary camel ovaries at 4 or 20°C for different periods of time on the morphology and viability of pre-antral follicles

Wpływ przechowywania jajników wielbłąda jednogarbnego
w temperaturze 4 lub 20°C przez różne okresy
na morfologię i żywotność pęcherzyków przedzatokowych

Summary. The purpose of this study was to evaluate the effect of cooling ovarian tissue on camel pre-antral follicles. 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 morphologically and viability evaluated by trypan blue exclusion test. The percentage of morphologically normal and live growing follicles was significantly reduced in ovarian tissue stored at 20°C for 12, 18 or 24 h. To assessment the viability of stored follicles, 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. COCs were incubated for 30 h at 38.5°C under 5% CO_2 in humidified air. Follicles stored in either condition presented the same growth pattern in vitro as fresh follicles. 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 of growing follicles or their ability to mature *in vitro*.

Key words: camel, viability, pre-antral follicles, storage, 4ºC

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. Of all the oocytes within an ovary, over 90% are enclosed in pre-antral follicles [Saumande 1991]. In the last decades, many studies have been carried out focusing on this great population of ovarian follicles. Various methods have been developed to isolate and culture pre-antral follicles from cow [Gutierrez *et al.* 200]), Buffalo [Abd-Allah

2009], 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 these follicles in several species.

The short-term preservation is especially important for the transportation of ovaries, 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 the different species, the results of all these works were similar. In general, the most suitable temperature was 4°C, allowing the good preservation of pre-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 pre-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 pre-antral follicles differ from fully grown oocytes in several structural and functional aspects [Shaw *et al*. 2000]. These differences make preantral 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 lipid is found in these oocytes [Shaw *et al*. 2000]. If camel pre-antral follicles can be successfully preserved under low temperatures, as pre-antral follicles from other species can, the use of these follicles would be a good alternative for the preservation of camel oocytes.

The present study aimed to evaluate the effect of storing pre-antral follicles within camel ovaries under different temperatures, and to test the ability of these follicles to grow *in vitro* after being stored. The temperatures of 4 and 20°C were chosen based on the results of previous works [Silva *et al*. 2000].

The optimal time for in vitro maturation of dromedary camel oocytes was 30 h [Abd-Allah *et al*. 2008]. However, other reports suggested that it was 36 h [Torner *et al.* 2003].

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, TB stain is a useful and quick method to arrest the initial quality and viability of follicles [Jewgenow and Goritz 1995, Abd-Allah *et al*. 2008].

MATERIAL AND METHODS

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

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, striped of fat tissue and ligaments and rinsed in saline solution.

Of all the ovaries collected, 100 ovaries were not stores as control and the remaining ovaries (200) were divided into two groups, the first group was stored at 4°C and other group was stored at 20°C for 6, 12, 18 and 24 h, comprising the 2 groups. The temperature was maintained with the use of a fridge (4°C) and an electric thermo flask (20°C) and measured at the beginning of the experiment. Each treatment was repeated 5 times.

Examination of the morphology of the oocytes

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

The 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 granulosa- enclosed oocytes (whenever there were granulosa cell- free regions on the oocyte surface) and C^{-} for granulosa- free oocytes.

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

Examination of oocyte viability 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) and the staining of oocytes was performed at room temperature [Abd-Allah *et al*. 2008].

The ovary was used to recover immature oocytes from antral follicles. The immature oocytes were isolated in Dulbecco PBS (DPBS), washed in culture media and a final cell suspension was made in culture media (1 ml) in preparation for use both in a trypan blue exclusion test [Freshney 2000]. The exclusion test was used to provide an assessment of cell membrane integrity (only using those cells with damaged or non- intact cell membranes).

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 viable and fully stained oocytes as dead [Abd-Allah *et al*. 2008].

***In vitro* maturation of camel oocytes**

Follicle evaluation during and after *in vitro* culture. When growing follicles isolated from ovaries stored at 4°C for 24 h and at 20°C for 6 h were placed into culture, they grew *in vitro* in the same pattern as fresh follicles did.

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 post-stored COCs was conducted with mineral oil, for an incubation time (30 h) at 38.5°C and in an atmosphere of 5% CO_2. At the end of incubation period, COCs were examined for cumulus expansion and first polar body for assessment of maturation.

Data were analyzed using Chi-square analysis [Snedecor and Cochran 1980].

RESULTS

Oocytes morphology and viability

A total of 1140 COCs were recovered from 300 ovaries (average of 3.8/ ovary). Percentages for oocyte classes of recovered oocytes and normal morphology in addition live camel oocytes after storing ovaries at 4ºC or 20ºC at different periods are presented in Tab. 1 and Fig. 1, 2.

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

Time, h	Storing temp, °C	Recovered oocytes			Morphological observation			Trypan blue exclusion test		
		C^+	$C^{+/-}$	C^-	C^+	$C^{+/-}$	C^-	C^+	$C^{+/-}$	C^-
0	4	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)
	20	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	4	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)
	20	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	4	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)
	20	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	4	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)
	20	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% (2/10)	16% (8/50)
24	4	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)
	20	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] (1/8)	0.0% [c] (0/4)	9% [c] (6/66)

Values with the same superscript in the same column were significantly different at P greater than 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.

The percentage of morphologically normal granulosa- enclosed oocytes derived from pre-antral follicles of stored ovaries at 4ºC increased significantly ($p < 0.05$) in comparison to that of stored ovaries at 20º C and the percentage of granulosa- free oocytes derived from stored ovaries at 20ºC increased significantly ($p < 0.05$) in comparison to that of stored ovaries at 4º C (Fig. 2).

As shown in Table 1, 72–91.8% and 63.6–85.7% of the follicles from stored ovaries at 4ºC and stored ovaries at 20ºC presented normal morphology on 0 h and live oocytes (Tab. 1), and no significant differences were observed among groups.

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

Criteria	Recovered good oocytes $C^{+}/C^{+/-}$	Normal oocytes $C^{+}/C^{+/-}$	Live oocytes $C^{+}/C^{+/-}$	Maturation rate $C^{+}/C^{+/-}$
Non-stored ovaries	85.5% [a] (65/76)	89.2% [a] (58/65)	87.0% [a] (54/62)	85% [a] (46/54)
Ovaries stored at 4ºC for 24 h	82.0% [a] (64/78)	81.2% [a] (52/64)	81.2% [a] (52 /64	80.0% [a] (42/52
Ovaries stored at 20ºC for 6h	73.7% [a] (59/80)	79.6% [a] (47/59)	76.2% [a] (45/59)	77.7% [a] (35/45)

Within the same column, values with the same superscript are insignificantly different from each other ($P > 0.05$)

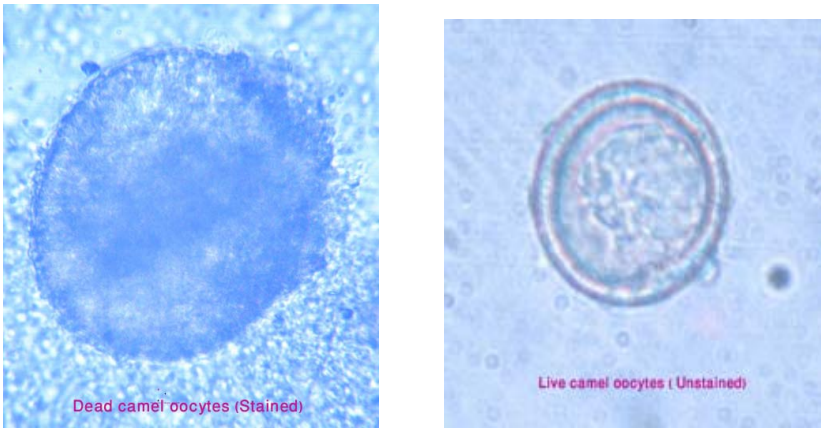

Fig. 1. Camel oocytes stained with trypan blue: dead camel oocytes (stained) and live camel oocytes (unstained)

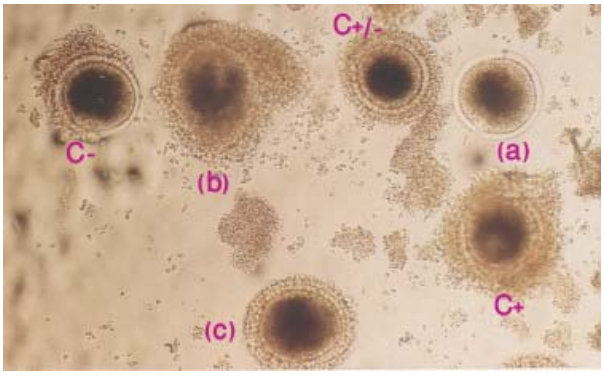

Fig. 2. Morphologically normal and abnormal camel oocytes retrieved from stored ovaries at 4ºC for 24 h, scored for granulose-oocyte adhesion: C^{+} granulosa-enclosed oocytes, $C^{+/-}$ partially granulosa-enclosed oocytes (whenever there were granulosa cell-free regions on the oocyte surface), C^{-} granulosa-free oocytes, (a) Rupture of zona pellucida, (b) Abnormal size of oocyte, (c) Asymmetrical shape of oocyte

Ovarian storage at 20ºC for 12, 18 or 24 h significantly reduced ($P < 0.05$) the percentage of morphologically normal and live growing follicles compared with the stored ovaries at 4ºC for 24 (Tab. 1 and Fig. 2). The percentage of normal and live growing follicles in ovarian pieces stored at 20ºC for 12 and 18 h was also significantly lower than in ovarian tissue stored at the same temperature for 6 h ($P < 0.05$). In addition, the temperature of 20ºC proved to be inferior to 4ºC in preserving the morphology of growing follicles when ovarian tissue was stored for 12, 18 or 24 h, and resulted in significantly reduced percentages of normal and live growing follicles ($P < 0.05$).

In vitro maturation

On 30 h of culture (Tab. 2 and Fig. 3), 85%, 80% and 77.7% of the follicles from fresh ovaries, ovaries stored at 4ºC for 24 h and ovaries stored at 20ºC for 6 h, respectively were matured and non significantly different ($P < 0.05$).

DISCUSSION

This study shows for the first time that camel pre-antral follicles can be successfully stored in ovaries at low temperatures. The percentage of normal and live growing follicles was significantly reduced in ovarian tissue stored at 20°C for 12 18 or 24 h. The growing follicles have already started their development. They present many granulosa cells in mitotic activity. 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 follicles 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. On the other hand, the temperature of 4°C probably lowered the cellular metabolism, slowing down the degenerative process. These results are comparable to those reported for cow [Lucci *et al.* 2004] and goat [Silva *et al*. 2000] pre-antral follicles stored at the same temperatures.

Morphological assessment of follicular integrity has been largely used to evaluate the effectiveness of the various treatments to which ovarian follicles are submitted [Silva *et al*. 2000, Lucci *et al*. 2004]. However, the ability to progress on the development is the most reliable evaluation of follicles/oocytes viability.

After storage at 4°C for 24 h or at 20°C for 6 h, growing follicles were able to continue developing when cultured for 30 h, and presented a growing pattern similar to fresh follicles.

Camel pre-antral follicles from fresh and stored ovaries grew at a maturation rate of 77.7–85% on 30 h of culture. These results are similar to those described by Abd-Allah *et al.* [2008] for fresh camel follicles.

After culture, a variable percentage of follicles showed signs of degeneration (Tab. 1). A variable number of follicles were expected to degenerate during in vitro culture [Telfer *et al.* 2000]. The culture conditions themselves, which are not well established yet, can partially explain the degeneration of follicles, although the factors that lead to degeneration are many. In follicles from fresh ovaries and ovaries stored at 4ºC for 24 h or at 20ºC for 6 h, the degenerative signs usually consisted on a misshapen oocyte with

a coagulated cytoplasm. Degeneration of the oocyte is the most common sign of degeneration observed in cultured follicles [Figueiredo *et al*. 1994].

The results of this study also show that camel oocytes enclosed in pre-antral follicles are not as cold sensitive as fully-grown oocytes. This may be attributed to the cytoplasmic droplets are considered to play important roles in energy metabolism during oocyte maturation and to be responsible for the oocytes cooling intolerance [Nili *et al*. 2004]. Changes in both the morphology and amount of cytoplasmic lipid droplets have also been demonstrated during camel oocyte maturation [Nili *et al*. 2004].

CONCLUSION

The storage of ovaries at 4°C for up to 24 h did not affect the percentage of morphologically normal growing follicles, while ovarian storage at 20°C for 12, 18 or 24 h reduced the percentage of morphologically normal growing follicles. Moreover, storage of camel ovaries at 4°C for up to 24 h or at 20°C for up to 6 h does not affect the morphology of growing follicles or their ability to mature *in vitro*. The results presented here confirm that camel oocytes enclosed in pre-antral follicles are not sensitive to low temperatures. The use of pre-antral follicle oocytes can be a good alternative for the preservation of camel germinal cells.

REFERENCES

Abd-Allah S. M., 2009. *In vitro* production of buffalo embryos from stepwise vitrified immature oocyte s. Vet. Italiana, 45(3), 425–429.

Abd Allah S.M., Khalil A.A.Y., Ali K.M., 2008. The use of trypan blue staining to select the developmentally competent dromedary camel oocytes and its effect on *in vitro* maturation rate. Proc. Twentieth Annual Scientific Conference, 2–7 February, Fayoum. Egyptian Society of Reproduction and Fertility, 207–218.

Candy C.J., Wood, M.J., Whittingham D.G., 1997. Effect of cryoprotectants on the survival of follicles in frozen mouse ovaries. J. Reprod. Fertil. 110, 11–19.

Combelles C., Albertini D., 2003. Assessment of oocyte quality following repeated gonadotropin stimulation in the mouse. Biol. Reprod. 68, 812–821.

Cury-Boaventura M.F., Gorja˜o R., Lima T.M., Newsholme P., Curi, R., 2006. Comparative toxicity of oleic and linoleic acid on human lymphocytes. Life Sci. 78, 1448–1456.

Fair T., Hulshof S.C.J., Hyttel P., Greve T., Boland M., 1997. Nucleus ultrastructure and transcriptional activity of bovine oocytes in preantral and early antral follicles. Mol. Reprod. Dev. 46, 208–215.

Figueiredo J.R., Hulshof S.C.J., Van Den Hurk R., Nusgens B., Bevers M.M., Ectors F.J., Beckers J.F., 1994. Preservation of oocyte and granulosa cell morphology in bovine preantral follicles cultured *in vitro*. Theriogenology 41, 1333–1346.

Freshney R.I., 2000. Culture of animal cells: a manual of basic techniques, 4th ed. Wiley-Liss Inc., New York, 187–196.

Gosden R.G., 2000. Low temperature storage and grafting of human ovarian tissue. Mol. Cell. Endocrinol. 163, 125–129.

Gutierrez C.G., Ralph J.H., Telfer E.E., Wilmut I., Webb R., 2000. Growth and antrum formation of bovine preantral follicles in long-term culture *in vitro*. Biol. Reprod. 62, 1322–1328.

Hyttel P., Fair T., Callensen H., Greve T., 1997. Oocyte growth, capacitation and final maturation in cattle. Theriogenology 47, 23–32.

Jewgenow K., Goritz F., 1995. The recovery of preantral follicles from ovaries of domestic cats and their characterization before and after culture. Anim. Reprod. Sci. 39, 285–297.

Lucci C.M., Kacinskis M.A., Rumpf R., Ba´o S.N., 2004. Effects of lowered temperatures and media on short-term preservation of zebu (*Bos indicus*) preantral ovarian follicles. Theriogenology 61, 461–472.

Nili H., Mesbah F., Kafi M., Nasr Esfahani H., 2004. Light and transmission electron microscopy of immature *Camelus dromedariuc* oocytes. Anat. Histol. Embryol. 33, 196–199.

Saumande J., 1991. La folliculoge´ne`se chez les ruminants. Rec. Me´d. Ve´ t. 167, 205–218.

Shaw J.M., Oranratnachai A., Trounson A.O., 2000. Fundamental cryobiology of mammalian oocytes and ovarian tissue. Theriogenology 53, 59–72.

Silva J.R.V., Lucci C.M., Carvalho F.C.A., Ba´o SN., Costa S.H.F., Santos R.R., Figueiredo J.R., 2000. Effect of coconut water and Braun-Collins solutions at different temperatures and incubation times on the morphology of goat preantral follicles preserved *in vitro*. Theriogenology 54, 809–822.

Snedecor G.W., Cochran W.F., 1980. Statistical methods. 7th ed. Iowa State University Press, Ames, Iowa, pp. 508.

Telfer E.E., Binnie J.P., McCaffery F.H., Campbell B.K., 2000. *In vitro* development of oocytes from porcine and bovine primary follicles. Mol. Cell. Endocrinol. 163, 117–123.

Streszczenie. Celem niniejszego badania była ocena wpływu chłodzenia tkanki jajników na przedzatokowe pęcherzyki wielbłąda. Jajniki przechowywano w roztworze soli (0,9%) w temperaturze 4 lub 20^{o}C przez 6, 12, 18 lub 24 h. Następnie COC odzyskiwano z jajników wielbłąda i oceniano je pod względem morfologii i żywotności przy użyciu błękitu trypanu. Procent morfologicznie normalnych i żywych rosnących pęcherzyków był istotnie zmniejszony w tkance jajników przechowywanej w temperaturze 20°C przez 12, 18 lub 24 h. Aby ocenić żywotność przechowywanych pęcherzyków, morfologicznie normalne i żywe COC wyizolowane z jajników przechowywanych w temperaturze 4°C przez 24 h oraz 20°C przez 6 h poddano dojrzewaniu *in vitro* (IVM) w TCM 199 + 10 µg/ml FSH + 10 IU hCG/ml + 10% FCS + 50 µg/ml gentamycyny. COC inkubowano przez 30 h w temperaturze 38,5°C w warunkach 5% CO_2, w nawilżonym powietrzu. Pęcherzyki przechowywane w różnych warunkach wykazały taki sam model wzrostu *in vitro* jak świeże pęcherzyki. Przechowywanie jajników wielbłąda w temperaturze 4°C do 24 h lub w temperaturze 20°C do 6 h nie wpłynęło na morfologię rosnących pęcherzyków ani na ich zdolność do dojrzewania *in vitro*.

Słowa kluczowe: wielbłąd, żywotność, pęcherzyki przedzatokowe, przechowywanie, 4ºC