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Successful cryopreservation of buffalo ovaries using *in situ* oocyte cryopreservation

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Summary

To improve the efficiency and efficacy of cryopreservation of ovaries, we developed a new method termed *in situ* oocyte (ISO) cryopreservation. ISO cryopreservation is a multistep procedure that involves aspiration of follicular fluid and then perfusion of antral follicles and diffusion of whole buffalo ovaries with cryoprotectant agent (CPA), rapid cooling, storage, thawing and, finally, dilution and removal of the CPA with return to physiological environment. Our study compared ISO cryo ovaries with cryo-diffused ovaries. We systematically examined the effects of ISO cryo and diffuse cryo on ovaries by morphological examination and with viability tests. The percentages of morphologically normal and viable follicular oocytes from ISO cryo were significantly higher than those that resulted from the cryo-diffused method ($p<0.01$). The quality of follicular oocytes from ISO cryo ovaries appeared better than that achieved from cryo-diffused ovaries. In conclusion, this study shows that ISO cryo is highly efficient for cryopreservation of oocytes and ovarian tissue.

Keywords

Buffalo, Cryopreservation, Egypt, *In situ*, ISO, Oocyte, Ovary.

Crioconservazione di successo di ovaie bufaline con il metodo della crioconservazione degli oociti *in situ*

Riassunto

Allo scopo di dimostrare l'efficacia e l'efficienza della crioconservazione delle ovaie, è stato sviluppato un nuovo metodo denominato: in situ *oociti (ISO) crioconservazione. Il metodo si avvale di una procedura multifase che comporta: aspirazione del fluido follicolare, successiva perfusione dei follicoli antrali e diffusione di tutte le ovaie del bufalo con agente crioconservante (CPA), rapido rinfrescamento, accumulo, scongelamento e, infine, diluizione e rimozione del CPA con ritorno all'ambiente fisiologico. Il presente studio mette a confronto la ISO crioconservazione delle ovaie con la criodiffusione delle ovaie. Sono stati sistematicamente esaminati gli effetti della ISO crioconservazione e della criodiffusione sulle ovaie mediante esami morfologici e test di vitalità. Le percentuali di oociti morfologicamente nella norma e con follicoli vitali sono risultate significativamente maggiori nel caso del metodo ISO crioconservazione rispetto al metodo della criodiffusione ($p<0.01$). La qualità degli oociti follicolari delle ovaie con il metodo della ISO crioconservazione è apparsa migliore rispetto a quella ottenuta con il metodo della criodiffusione ovarica. In conclusione lo studio mostra come il metodo della ISO crioconservazione sia più efficiente per la crioconservazione degli oociti e del tessuto ovario.*

Parole chiave

Bufalo, Crioconservazione, Egitto, *In situ* Oociti (ISO), Ovaie.

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Introduction

Recent research investigating the *in situ* oocyte (ISO) cryopreservation of follicular oocytes has primarily been focused on the improvement of cryopreservation methods of immature oocytes to overcome cryoinjury and to regulate the cumulus-oocyte interface.

ISO cryo is potentially a useful technology for the preservation of genetic resources of experimental, domestic and wild animals.

To achieve optimal cryoprotection, it is essential that freezing protocols allow uniform penetration of cryoprotectant agent (CPA) throughout the ovarian tissue. Thus, the rate of CPA permeation is an important determining factor in developing better cryopreservation protocols for ovarian tissues. To date, several factors have been determined that contribute to the development of optimal cryopreservation protocols for oocytes (6).

Although whole ovaries from mice and rats survive freezing because of their smaller sizes, in these species effective cryoprotectant penetration can occur through simple diffusion. This is not the case for larger species whose ovaries are larger and more fibrous (2, 20). Therefore, a technique of ISO cryo needs to be adopted where the cryoprotectant is perfused though the ovary via the antral follicles as well as diffusion of CPA in the entire ovary.

During the cryopreservation procedure, cells and tissues undergo volume changes due to different osmotic pressures between the intracellular and extracellular solutions (16). These changes in cell volume affect several parameters that play a role in the cryosurvival of oocytes, including integrity of the plasma membrane and subcellular organelles (3, 14).

Methods developed for oocyte and ovarian tissue cryopreservation must protect structural and functional viability. The strategy describes cryopreservation of oocytes at their location within the antral follicle.

Trypan blue stain has been used previously to detect oocyte viability (1, 9) and viability of follicles in cryopreserved human ovarian tissue (8).

The trypan blue exclusion test is based on plasma membrane integrity (17). Dead oocytes displayed a dark blue ooplasm with translucent cumulus cells. Moreover, it has been reported that the trypan blue stain is a useful and rapid method to arrest the initial quality and viability of follicles (5, 12).

However, there were no reports available on the ISO cryopreservation of buffalo oocytes. Therefore, the present study was conducted to compare the influence of the ISO cryopreservation and cryo-diffused methods on the successful cryopreservation of buffalo ovaries as a potential model for human ovary cryopreservation.

Materials and methods

All materials were purchased from the Sigma Chemical Company (St Louis, Missouri) unless otherwise indicated.

Experimental design

Ovaries were obtained from buffalo that had just been slaughtered at a local abattoir for each experiment. Each buffalo was on average 5-15 years of age.

Experiment I (cryo diffusion)

Freshly collected ovaries with an antral follicle (Fig. 1) were dissociated from adipose tissue and transferred to a Petri dish containing 10% glycerol solution at 37°C. After a 30 min exposure to the 10% glycerol solution, each ovary was transferred to the 20 ml/cc mediject syringe (Intermedica, Cairo) containing the 10% glycerol solution (15). The cryo syringes were plunged directly into liquid nitrogen (–196°C) for vitrification of the ovary and were stored for a month.

Experiment II (the *in situ* oocyte cryopreservation)

The antral follicle (10-12 mm in diameter) was aspirated by a hypodermic needle 26 gauge (G) × 1/2″ (0.45 × 12 mm) (Intermedica, Cairo) to remove part of the follicular fluid (2-5 mm^3) and then the follicle was infused by 10% glycerol (2-5 mm^3) and each ovary was utilised as described above.

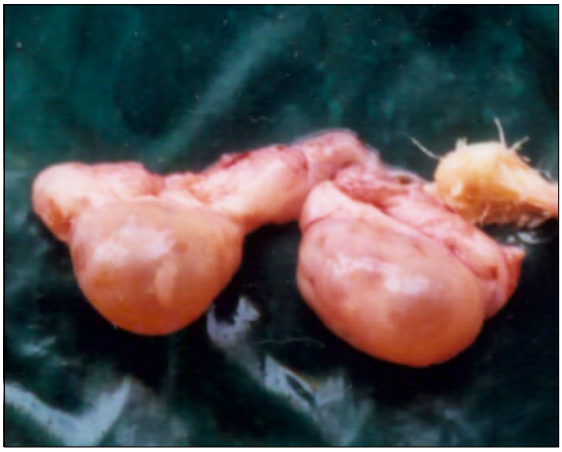


Figure 1
Freshly collected buffalo ovaries with antral follicles utilised in cryopreservation

Thawing of ovaries

In both experiments, the cryo syringes were removed from the liquid nitrogen and held at room temperature for 2 min before being plunged into a 37°C water bath and gently agitated for 30-40 min. Each ovary was immersed, through the needle of the cryo syringe, into a thawing media consisting of tissue culture medium-199 (TCM-199) supplemented with 10% foetal calf serum (FCS), 50 µg/ml gentamycine sulfates, 10% sucrose and, through the hypodermic needle 18 G × 1 1/2″ (0.8 × 40 mm) attached to a 10 ml/cc mediject syringe (Intermedica, Cairo) containing warm (37°C) thawing media aspirate buffalo immature oocytes from antral follicles (10-12 mm in diameter) and other growing follicles (2-8 mm in diameter). The ovaries were kept at 37°C in the final thawing media until analysis.

Survival of oocytes after vitrification-thawing

Examination of the morphology of the oocytes

The number of buffalo oocytes retrieved from antral follicles and growing follicles was recorded for each experiment.

The released immature buffalo oocytes were scored for granulose-oocyte cell adhesion as previously described (4):

- C^{+} for granulosa-enclosed oocytes
- $C^{+/-}$ for partially granulosa-enclosed oocytes (whenever there were granulosa cell-free regions on the oocyte surface)
- C^{-} for granulosa-free oocytes.

The post-thawing survival of oocytes was observed under a stereomicroscope (M6C-9, 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 (7).

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 (1, 11).

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 (10). 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 categorised on the basis of the degree of dye exclusion. Unstained oocytes were classified as viable and fully stained oocytes as dead (1, 13).

Statistical analysis

The experiment was replicated 10 times and the data were analysed using the Chi-square analysis (18).

Results

Morphology of the oocytes

Percentages for oocyte classes and normal morphology of buffalo oocytes after cryopreservation by ISO or diffusion are presented in Table I and in Figures 2, 3 and 4.

Table I
Number and percentages of morphologically normal and viable oocytes retrieved from antral and growing follicles in the groups of *in situ* oocyte cryopreservation and diffused cryopreserved ovaries, scored for granulose-oocyte adhesion

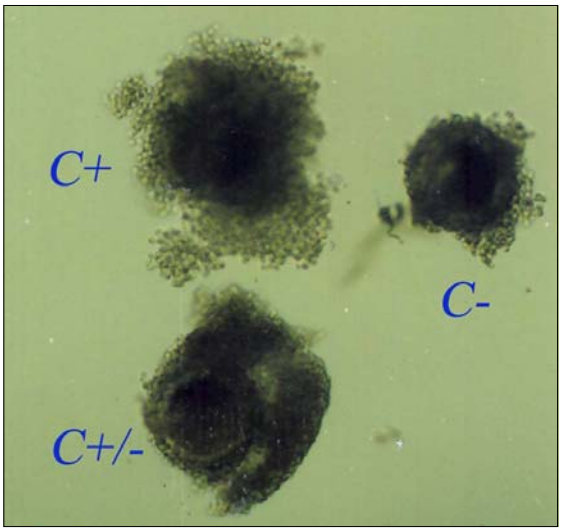
Criteria	Oocyte class (number and percentage)			Morphological observation[a]		Trypan blue exclusion test[b]	
	Class	ISO cryo ovaries	Diffused cryo ovaries	ISO cryo ovaries	Diffused cryo ovaries	ISO cryo ovaries	Diffused cryo ovaries
Antral follicles	C^{A+}	140 (70%)[c]	50(25%)[c]	130(92.8%)[d]	22(44%)[d]	126(90%)[e]	20(40%)[e]
	$C^{A+/-}$	50(25%)	30(15%)	40(80%)[d]	12(40%)[d]	36(72%)[e]	8(26.6%)[e]
	C^{A-}	10 (5%)[c]	120(60%)[c]	6(60%)[d]	40(33.3%)[d]	4(40%)[e]	26(21.6%)[c]
Growing follicles	C^{a+}	240(60%)[c]	80(20%)[c]	220(91.6%)[d]	30(37.5%)[d]	210(87.5%)[e]	24(30%)[e]
	$C^{a+/-}$	120 (30%)	80 (20%)	90(75%)[d]	28(35%)[d]	74(61.6%)[e]	20(25%)[e]
	C^{a-}	40 (10%)[c]	240 (60%)[c]	22(55%)[d]	48(20%)[d]	14(35%)[e]	40(16.6%)[e]

a) morphologically normal oocytes
b) viable oocytes

ISO cryo *in situ* oocyte (ISO) cryopreservation

Values with the same superscript c), d), e) in the same row were significantly different at $p>0.01$

C^{+} granulosa-enclosed oocytes
$C^{+/-}$ partially granulosa-enclosed oocytes (whenever there were granulosa cell-free regions on the oocyte surface)
C^{-} granulosa-free oocytes
C^{A-} suffix for oocytes retrieved from antral follicles
C^{a-} suffix for oocytes retrieved from growing follicles



C^{+} granulosa-enclosed oocytes
C+/– partially granulosa-enclosed oocytes (whenever there were granulosa cell-free regions on the oocyte surface)
C– granulosa-free oocytes

Figure 2
Morphologically normal oocytes retrieved from antral and growing follicles in the groups of *in situ* oocyte cryopreservation ovaries, scored for granulose-oocyte adhesion

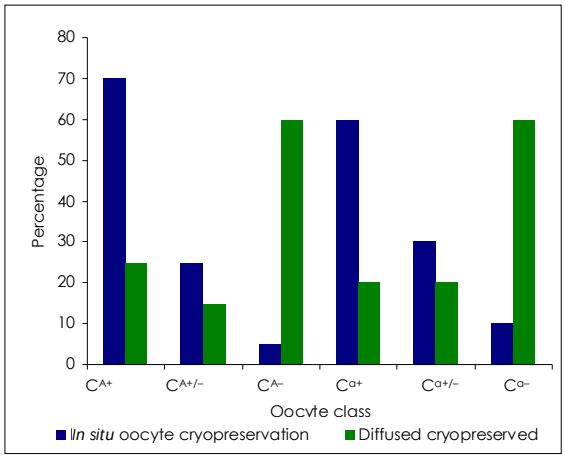


Figure 3
Percentages of oocyte class of antral and growing follicles retrieved from *in situ* oocyte (ISO) cryopreservation and diffused cryopreserved ovaries

Following the recovery of the oocytes, ISO cryo ovaries yielded granulosa-enclosed oocytes (70% from antral follicles and 60% from growing follicles) which is higher than the percentages recovered from controls (25% and 20%, respectively) ($p<0.01$).

The percentage of morphologically normal granulosa-enclosed oocytes derived from antral follicles cryopreserved by ISO increased significantly ($p<0.01$) in comparison to that of diffused cryopreserved ovaries and the percentage of granulosa-free oocytes derived

from diffused cryopreserved increased significantly (p<0.01) in comparison to that of ISO cryo ovaries (Fig. 4).

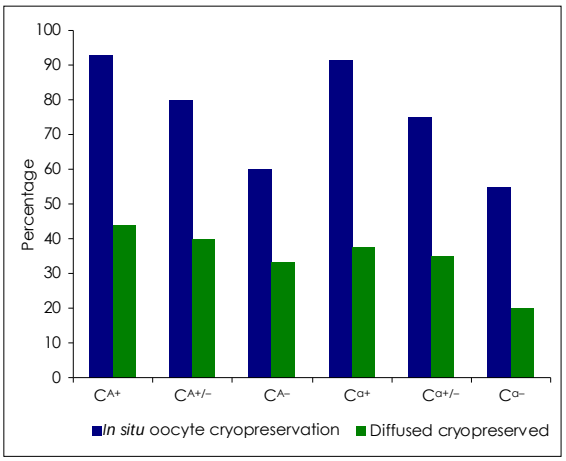


Figure 4
Percentages of morphologically normal oocyte class of antral and growing follicles retrieved from ISO and diffused cryoperserved ovaries

Viability of oocytes

The percentage of viable granulosa-enclosed oocytes derived from antral follicles cryopreserved by ISO increased significantly (p<0.01) in comparison to that of diffused cryopreserved ovaries and the percentage of granulosa-free oocytes derived from diffused cryopreserved increased significantly in comparison to that of ISO cryo ovaries (p<0.01) (Table I).

Discussion

The number and percentages of morphologically normal and viable granulose-enclosed oocytes retrieved from ISO cryo ovaries were significantly higher than those of the groups of diffused ovaries (p<0.01). This may be attributed to the ISO cryo that may prevent some of the apoptosis which can be induced by cryopreservation (19), as CPAs perfused directly to the follicular tissue would maximise cooling to facilitate vitrification and prevent ice crystal injury; it also resulted in less ultrastructural injury and hence improved tissue survival.

ISO cryopreserving of buffalo oocytes is necessary from a cryobiology point of view because the rate of CPA/cellular water exchange is affected by the amount of tissue through which the CPA must diffuse. During the cooling stage of cryopreservation, the relative distance of cells within the ovary from the exterior affects the rate at which these cells undergo cooling.

In the present study related to diffused cryopreserved ovaries, the percentages of morphologically normal and viable oocytes decreased significantly (p<0.01). This result was to be expected, probably due to inadequate cryoprotectant permeation in the antral follicle which may have compromised the oocyte's integrity during the cryopreservation protocol. Furthermore, possible intrafollicular ice formation at the antral cavity could have increased the oocyte lysis rate.

The number and percentage of morphological normal and viable granulosa-free oocytes derived from diffused cryopreserved increased significantly (p<0.01) in comparison to that of ISO cryo ovaries. This increase could have resulted from a shift of oocytes from the granulosa-enclosed classes to the granulosa-free oocytes. Two different mechanisms can be hypothesised to account for this cryo-induced damage namely:

- the increase in granulosa-free oocytes could result from the massive disruption of granulosa cell adhesion to the oocyte, denuding it directly to C–,
- a more gradual denuding mechanism could take place, with oocytes progressively losing their adhered granulosa cells to the intermediate stage (C+ /–) and, from this, to the denuded state (C–).

Both hypotheses are consistent with the observation that while C+ oocytes exhibit a significant post-thawing decrease, C+/– oocyte numbers are relatively unaffected by cryopreservation (although in the gradual denuding mechanism these oocytes would have derived from the C+ state).

Conclusion

It is concluded that, due to the high performance obtained, the ISO cryopreservation method used in this study is considered more suitable for the freezing of buffalo

oocytes and ovarian tissues than the cryodiffused method. Future investigations should include an *in vitro* fertilisation procedure to compare the successes in embryo development resulting from the two oocyte preservation methods.

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