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SUCCESSFUL PRODUCTION OF BUFFALO EMBRYOS FROM VITRIFIED IMMATURE AND MATURE OOCYTES IN VITRO

(With One Table and 4 Figures)

By

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**إنتاج ناجح لأجنة الجاموس من بويضات غير ناضجة وناضجة
مزججه بالتجميد السريع معمليا**

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تهدف الدراسة الحالية إلى إنتاج أجنة الجاموس معمليا من بويضات غير ناضجة وناضجة مجمدة سريعا. تم الحصول على البويضات المحاطة بالخلايا الوسادية من مبايض الجاموس المذبوحة محليا. تم تجميد البويضات الغير ناضجة مباشرة بعد سحبها من المبايض وجزء آخر تم تجميده بعد زرعها لمدة 22 – 24 ساعة. تم تجميد كلاهما باستخدام تركيزات مختلفة من الجليسرول (3.3 و 6.6 و 10 %) لمدة 5 و 5 و 10 دقائق بالترتيب و على التوالي و بعد ذلك تم وضع البويضات فى قصبيات 0.25 ملليمتر محتوية على 10% سكروروز ثم بعد ذلك حفظت فى سائل النتروجين لمدة 4-6 شهور. تم اذابة القصبيات فى حمام مائى لمدة 30 ثانية عند درجة حرارة 37 درجة مئوية. البويضات الغير ناضجة تم استكمال عملية زرعها لمدة 22-24 ساعة وكلاهما تم اخصابهما فى المعمل وبعد الاخصاب تم زراعة البويضات لمدة 6-7 ايام وتم تسجيل مرحلة الموريلا والبلاستوسيست لكل منهما. أظهرت النتائج ان نسبة البويضات السليمة بعد التجميد ذات فروق معنوية بين البويضات الغير ناضجة والناضجة (55.4% و 70.6%) وكذلك كانت أعلى نسبة حصول على أجنة من البويضات الناضجة عنها من البويضات الغير ناضجة (10.3% و 2.6% على التوالي).

SUMMARY

The current study was conducted to produce buffalo embryos in vitro from vitrified immature and mature oocytes. Cumulus oocytes complexes (COCs) were obtained from ovaries which collected from a local abattoir.

Good quality COCs (immature oocytes) were vitrified immediately after collection. After 22-24 hours of COCs culture, mature oocytes were vitrified similarly.

All oocytes (immature and mature) were exposed to vitrification solution of different concentrations (glycerol: 3.3, 6.6 and 10%) at 5, 5 and 10 minutes intervals respectively. The oocytes were finally placed in 10% glycerol and immediately loaded in 0.25 ml plastic straws, each containing 100 μ l of 10% sucrose in Modified phosphate buffer saline (M-PBS).

The straws were stored in liquid nitrogen for 4-6 months. The straws were thawed in water at 37°C for 30 seconds. After thawing, immature oocytes have been exposed to further maturation for 22-24 h. Mature oocytes were fertilized with frozen-thawed semen capacitated in modified fertilization TALP medium. After insemination, the oocytes were cultured for 6-7 days and the frequency of morula and/or blastocyst was recorded. Data analysis revealed that, the proportion of buffalo oocytes found to be normal after vitrification-thawing procedures was significantly different between immature and mature oocytes (55.4% and 70.6% respectively) and a significantly higher ($p < 0.05$) percentage of embryos development (morula and blastocyst) was derived from vitrified mature buffalo oocytes than those obtained from immature ones (10.3% Vs. 2.6%).

Key words: *Buffalo, Embryos, Cryopreservation, Oocytes*

INTRODUCTION

Cryopreservation method is a key factor in commercial embryo production and transfer technology, in addition offers the opportunity to implement novel animal breeding and production programs (Martinez, *et al.*, 1999).

Several cryopreservation methods such as conventional (slow), equilibrium rapid freezing (vitrification) and ultra rapid freezing have been used to preserve embryos and oocytes of many animal species resulting in the birth of live offspring (Kajihara *et al.*, 1992). Vitrification which is relatively recent approach defined as physical process by which a highly concentrated solution of cryoprotectants solidifies during cooling, without formation of ice crystals (Niemann, 1991). This offers several advantages over conventional equilibrium methods e.g. faster and simplified freezing and thawing procedures, high oocytes/embryos survival and no requirements for a freezing machine

(Dhali, *et al.*, 1999). Vitrification was reported to be suitable for cryopreservation of bovine oocytes (Hurr, *et al.*, 1999) and buffalo oocytes (Abd Allah 2003 and Badr *et al.*, 2008).

Badr, *et al.* (2008) examined freezing sensitivity of immature buffalo oocytes frozen by vitrification in different ethylene glycol concentrations (10, 20 and 40%) in the equilibration solution, equilibration periods (3, 5 and 10 min) and vitrification solutions (ethylene glycol with 0.5 M sucrose, 0.3 M trehalose and 20% dimethyl sulfoxide). Of all oocytes frozen, nothing had developed blastocysts.

The present study was conducted to produce buffalo embryos from vitrified immature and mature oocytes.

MATERIALS and METHODS

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

Collection of oocytes

During period of the study (from winter of 1998 till winter of 2003), buffaloes ovaries were collected from a local abattoir and transported to the laboratory within 3-4 h in warm (25°C) physiological saline containing 50 µg/µl gentamycin sulfate. COCs were recovered by aspiration of follicle 2-6 mm diameter using an 18 G needle attached to 10 ml syringe. Only oocytes with at least three layers of compact cumulus cells and homogenous cytoplasm were selected, as described by Ganguli, *et al.* (1998).

Experimental design

Buffalo oocytes were vitrified immediately after collection (immature oocytes) or vitrified after culture (mature oocytes, 24 h post-cultured). Fresh oocytes without vitrification undergoing the same maturation and fertilization protocols were used as control.

In vitro maturation

Selected COCs were washed three times with pre-equilibrated maturation medium (TCM-199) supplemented with 10% heat inactivated fetal calf serum (FCS), 50 µg/ml gentamycin sulfate. COCs with unexpanded cumulus cells and evenly granulated ooplasm were cultured in 50 µl drops (10 oocytes/droplet) of the same medium. The droplets were covered with sterile Millipore filtered mineral oil, pre-incubated under the maturation conditions for a minimum of 3 h (39°C, 5% CO₂ in air with 95% relative humidity) and then incubated for 22-24 h (Totey,

at al., 1993). The oocytes were assessed for maturation by cumulus mass expansion (Nandi, *et al.*, 2002).

Vitrification of oocytes

The vitrification solution consisted of different concentrations of glycerol (3.3, 6.6 and 10%) dissolved in M-PBS enriched with 10% FCS and antibiotics. At room temperature, the oocytes (either immature or mature oocytes) were suspended in vitrification solution in concentrations 3.3; 6.6 and 10 % in 3 steps at 5, 5, 10 minute's interval respectively. During equilibration for 5 minutes in the final solution (10% glycerol) about 10-15 oocytes were loaded in mini straws (0.25 ml) as previously described by Curtis (1991). The straws were sealed with hot sealer and pre cooled by keeping them in liquid nitrogen tank.

After storage for 4-6 month, the straws were warmed rapidly by transferring them to a water bath at 37°C for 30 seconds. The contents were expelled into an empty plastic sterile dish and the oocytes were allowed a 5 minutes equilibrium in 10% sucrose solution in M-PBS for one step dilution.

The oocytes were then transferred to fresh washing medium (TCM-199 plus 10% FCS with 50µg/µl gentamycin sulfate) and were washed 3 times with it. The frozen-thawed oocytes (immature) were further incubated for 24 h in CO₂ incubator under the same condition.

Survival of oocytes after vitrification-thawing procedures

Oocyte normality was evaluated according to Dhali, *et al.* (1999) by their post-thaw morphological appearance under stereo microscope. The criteria used for assessing morphology were as follow: normal oocytes with spherical and symmetrical shape with no sign of lysis, membrane damage, swelling, degeneration or leakage of the cellular content; abnormal oocytes with a ruptured zona pellucida or having fragmented cytoplasm with signs of degeneration.

In vitro fertilization:

Spermatozoa were prepared from frozen-thawed buffalo bull semen as described by Jaakma *et al.* (1995). The contents of two mini straws (0.25ml) were thawed in a water bath at 37°C for 30 seconds and pooled in a sterile warm tube. Thawed semen was layered, in six conical sterile tissue culture tubes, under 1.0 ml of modified sperm-Tyroid's albumin lactate pyruvate (mS-TALP; S-TALP supplemented with 200 IU/ml heparin sodium salt) medium for sperm capacitation. The tubes were held at angle of 45 degree for one hour at 39°C. The uppermost supernatant (200 µl) of each tube containing highly motile spermatozoa was obtained as well as pooled in a sterile 15 ml centrifuge tube and then

centrifuged at 1800 rpm for 10 minutes. The pelleted spermatozoa were re-suspended in a sufficient amount of modified fertilization-TALP (mF-TALP) to yield a final concentration of 2×10^6 sperm cells/ml.

Oocytes having expanded cumulus cells were washed 3 times in modified F-TALP medium. Then, placed in 50 μ l droplets of modified F-TALP (5 oocytes/droplet) and covered with warm sterile Millipore filtered mineral oil and incubated for one hour under the same gas atmosphere as for IVM. Insemination was then performed using 2 μ l/droplet of capacitated sperm. The co-cultured oocytes were incubated at 39°C, 5.0% CO₂ and 95% relative humidity during 24 h for fertilization.

The presumptive zygotes were denuded of surrounding cumulus cells by washing 3 times using TCM-199 medium supplemented with 10 % FCS, 50 μ g/ml gentamycin sulfate before transferring to droplets of the same supplemented medium (5 oocytes/100 μ l) and incubated in 5% CO₂ incubator for 48 h. Fertilization was indicated by the appearance of the second polar body and confirmed by cleavage of the oocytes to 2-4 cells after 48 h of insemination (Hensleigh and Hunter, 1985).

In vitro culture

Embryo culture took place in supplemented TCM-199 under mineral oil in a humidified atmosphere of 5% CO₂ at 39°C (Hammam, *et al.*, 1997) for 7 successive days with change of the medium every 48 h. Cleavage was assessed after 72 h of culture and the number of embryos developing to the morula and blastocyst stages was assessed on 6-7 days (Saeki *et al.*, 1990).

Statistical analysis

Data were analyzed by chi-square analysis (Snedecore and Cochran, 1980).

RESULTS

The results of the present study (Table 1 and Fig.1, 2) revealed that the percentages of post thawing morphologically normal vitrified mature oocytes were significantly higher ($P < 0.05$) than that of vitrified immature oocytes (70.6% vs. 55.4%).

The values of the cleavage and buffalo embryos rates for vitrified mature oocytes were significantly higher ($P < 0.05$) when compared with those of vitrified immature oocytes (33% and 10.3% vs. 13.3% and 2.6%, respectively).

Statistical analysis (Table 1) revealed that the presence of significant differences in the maturation and cleavage rates ($P<0.05$) in vitrified immature oocytes than that of fresh one (49.3%, 13.3% vs. 81.2%, 41.8%).

The differences between the percentages of developed embryos for vitrified immature (Fig.3) and fresh immature oocytes was highly significant ($P<0.05$), (2.6% vs. 11.3%). However, the values of buffalo embryos for vitrified matured oocytes (Fig.4) were not different significantly when compared with those for the fresh immature ones (10.3% vs. 11.5%).

Table 1: Survival rates and developmental competence of fresh and vitrified immature and mature oocytes after thawing.

Criteria	Fresh oocytes (Control)	Vitrified oocytes	
		Immature	Mature
Frozen oocytes: Total No.	-	560	340
Morphological normal (Post thawing) Total No. (%)	- -	310 55.4 ^a	240 70.6 ^b
Cultured oocytes Total No.	213	300	212
Matured oocytes Total No. (%)	173 81.2 ^a	148 49.3 ^b	- -
Cleaved oocytes Total No. (%)	89 41.8 ^a	40 13.3 ^b	70 33.0 ^a
Embryos Total No. (%)	24 11.3 ^a	8 2.6 ^b	14 10.3 ^a

In the same row, the values having different superscript letters are significantly different from control at $P<0.05$.

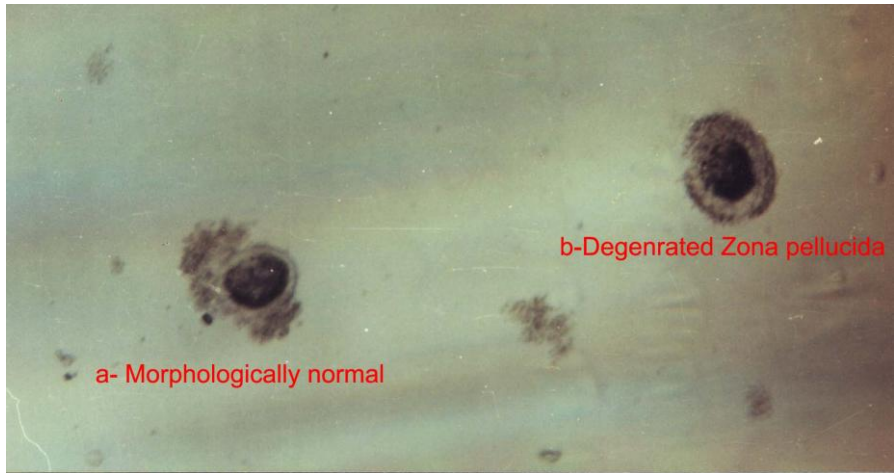


Fig. 1: (a) Morphologically normal post thawing vitrified immature oocytes
(b) Degenerated post thawing vitrified immature oocytes.

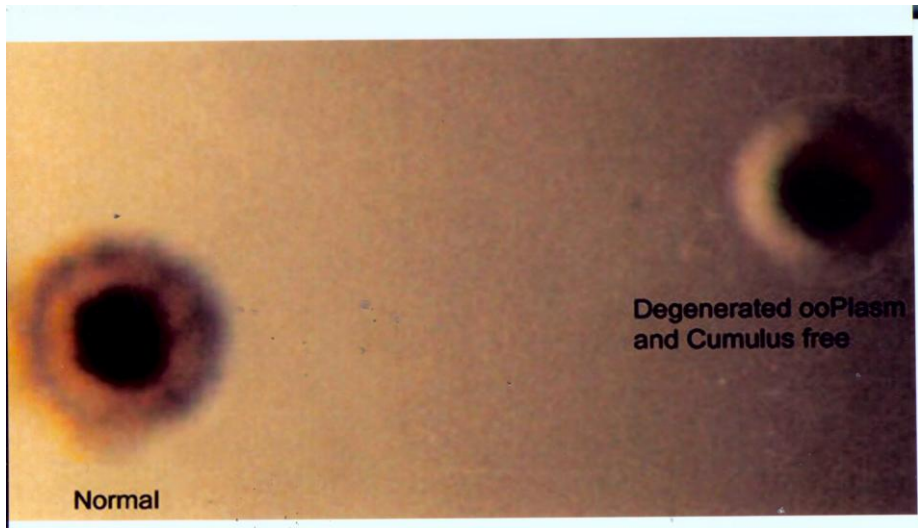


Fig. 2: Morphologically normal vitrified mature oocytes post thawing appeared and Degenerated vitrified mature oocytes post thawing.

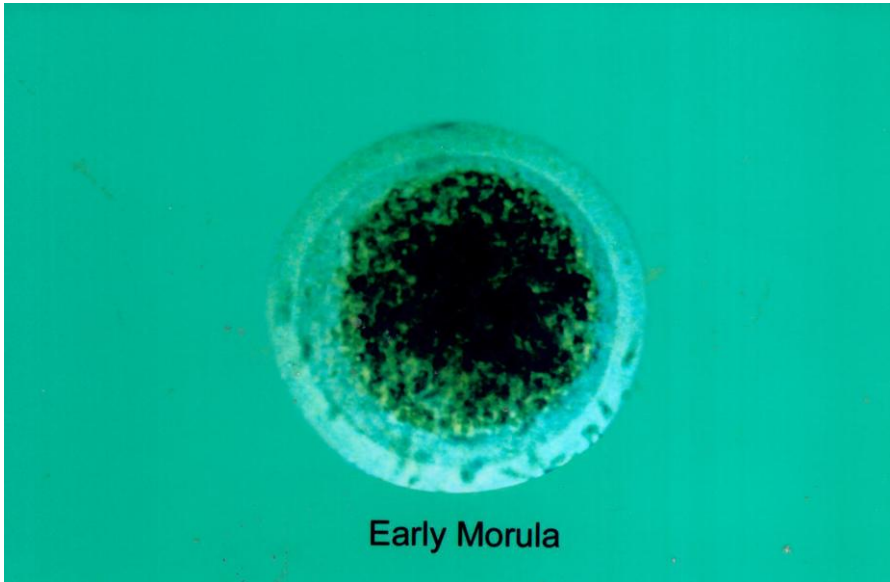


Fig. 3: Buffalo embryo obtained from vitrified immature oocyte.



Fig. 4: Buffalo embryo obtained from vitrified mature oocyte.

DISCUSSION

From the commercial point of view, cryopreservation technique of oocytes and embryos considered a very important factor for successful rate of embryo transferring.

The high survival rates of vitrified-thawed immature and mature buffalo oocytes observed in the present study compare favorably with other reports in which buffalo oocytes vitrified in 4.5 Mm ethylene glycol and 3.4 Mm dimethyle sulfoxide (Dhali, *et al.*, 1999). However, the maturation rate of buffalo oocytes in the present work in immature group was much lower after vitrification than that of fresh ones.

The proportion of post thawing morphologically normal vitrified mature oocytes were significantly higher ($P < 0.05$) than that of vitrified immature oocytes (70.4% vs. 55.4%).

A possible explanation for these results is that matured oocytes were found to be more resistant to freezing than immature ones (Fukui, *et al.*, 1992 and Lim, *et al.*, 1992), Moreover the hydrolyic conductivity of immature bovine oocytes is only half that of in vitro matured oocytes (Ruffing, *et al.*, 1993) and the permeability of bovine oocytes is significantly higher for cryoprotectant after in vitro maturation (Agea, *et al.*, 1997).

Data presented here also indicated that buffalo oocytes vitrified at the mature stage cleaved and developed into morula and blastocyst stage after thawing at a higher rate than those vitrified at the immature stage.

The freezability of immature oocytes has been reported to be low and increases as development proceeds to the blastocyst stage after fertilization (Kasai, *et al.*, 1979 and Schroeder, *et al.*, 1990).

The reduction in the fertilization may have resulted from disruption of the plasma membrane, extensive disorganization of the ooplasm, damage to the spindle or structural change in the zona pellucida during freezing (Carroll, *et al.*, 1990). In addition, freezing often results in low survival of bovine oocytes because of their extreme sensitivity to temperature near 0°C (Wu, *et al.*, 1998).

Data presented here indicated that vitrified immature buffalo oocytes developed into morula and blastocyst stage after thawing at a higher rate than those obtained by Badr, *et al.* (2008) who nothing had developed blastocysts. The differences between these findings may be due to IVF technique and in cryoprotectant concentration, duration of exposure to the cryoprotectant, and thawing procedure needed to be

adapted to the given stage of oocytes in order to obtain a good survival rate.

From the present study it could be concluded that the in vitro production of buffalo embryos could be obtained from vitrified immature and mature buffalo oocytes as already carried out by Abd Allah (2003).

REFERENCES

- Abd Allah, S.M. (2003):* In vitro fertilization, processing and cryopreservation of buffalo oocytes and embryos. Ph D. Theriogenology, Faculty of Veterinary medicine, Cairo University (beni-suef branch).
- Agea, Y.; Liu, J.; Peter, A.T.; Crister, E.S. and Crister, J.K. (1997):* Cryoprotectant and water permeability of immature and in vitro matured bovine oocytes. *Theriogenology*, 47 (1): 340(abstract).
- Badr, M.R.; Darwish, G.M. and El-Badry, D.A. (2008):* Cryopreservation of immature buffalo oocytes by vitrification. *Assiut Vet. Med. J.*, 54: 397-
- Carroll, J.; Depypere, H. and Mathews, C.D.(1990):* Freeze-thaw induced changes of the zona pellucida explain decreased rates of fertilization in frozen-thawed mouse oocytes. *J. Reprod. Fert.*, 90: 547-553
- Curtis, J.I. (1991):* Embryo transfer procedure. Academic press, Inc, UK, PP: 30-40.
- Dhali, A.; Manik, R.S.; Das, S.K.; Singla, S.K. and Palta, P. (1999):* Effect of ethylene glycol concentrations and exposure time on Post-vitrification survival and IVM rate of buffalo oocytes. *Theriogenology*, 50: 521-530.
- Fukui, E.; Kojima, T.; Shioya, Y.; Marcus, G.J. and Downey, B.R. (1992):* IVF and development of frozen-thawed bovine oocytes. *Cryobiology*, 29: 485-492.
- Ganguli, G., Indra, A. and Gupta, P. (1998):* Suitability of the follicular oocytes obtained from slaughtered buffalo ovaries and assessment of their nuclear maturation. *Buffalo J.* 2: 217-27.
- Hammam, A.M.; Zabaal, M.M. and Sabara, H.A. (1997):* Effect of types of media on IVM, IVC and IVF of buffalo and cattle oocytes *Beni – Suef, Vet. Med. Res.*, 2: 242-259.

- Hensleigh, H.C. and Hunter, A.G. (1985):* IVM of bovine cumulus enclosed primary oocytes and their subsequent IVF and cleavage. *J. Dairy Sci.*, 68: 1456-1462.
- Hurtt, A.E.; Squires, E.L. and Seidel, J.R. (1999):* Vitrification of equine and bovine oocytes in an ethylene glycol, ficoll and sucrose using open pulled straws. *Theriogenology*, 51: 166 (abstract).
- Jaakma, U.; Zhang, B.R.; Larsson, B.; Katska, L.; Rynska, B. and Smorag, Z. (1995):* The effect of different capacitation media on the in vitro fertilizability of bull spermatozoa. *Anim. Reprod. Sci.*, 44: 23-31.
- Kajihara, Y.; Kometani, N.; Shitanaka, Y.; Saito, S.; Yamaguchi, Y.; Hishiyama, K. and Endo, M. (1992):* Pregnancy rates and births after the direct transfer of frozen-thawed bovine IVF embryos. *Theriogenology*, 37, 233 (abstract).
- Kasai, M.; Kmi, J.H.; Takakamo, K.; Isudera, H.; Sakurai, T. and Machida, T. (1979):* A simple method for mouse embryo cryopreservation in a low toxicity vitrification solution, without appreciable loss of viability. *J. Reprod. Fertil.*, 89: 91-97.
- Lim, J.M.; Fuki, Y. and Ono, H. (1992):* Developmental capacity of bovine oocytes frozen at various maturation stages followed by IVM and IVF. *Theriogenology*, 37: 351-361.
- Martinez, A.G.; Brogliatti, G.M. and Lagomarsino, H.R. (1999):* Comparison of one step methods for freezing bovine embryos *Theriogenology*, 51: 145.
- Nandi, S.; Ravindranatha, B.M.; Gupta, P.S.P. and Sarma, P.V. (2002):* Timing of sequential changes in cumulus cells and first polar body extrusion during in vitro maturation of buffalo oocytes. *Theriogenology*, 57: 1151-1159.
- Niemann, H. (1991):* Cryopreservation of ova and embryos from livestock: current status and research needs. *Theriogenology* 35: 109-123.
- Ruffing, N.A.; Steponkus, P.L.; Pitt, R.E. and Park, J.E. (1993):* Osmotic behavior, hydrolytic conductivity and incidence of intracellular ice formation in bovine oocytes at different developmental stages. *Cryobiology*, 30: 562-580.
- Saeki, K.; Hoshi, M.; Leibfried-Rutledge, M.L. and First, N.L. (1990):* IVF and development of bovine oocytes matured with commercially available Follicle stimulating hormone. *Theriogenology*, 43: 1035-1039.

- Schroeder, A.C.; Champlin, A.K.; Mobraatin, I.E. and Eppig, J.J. (1990):* Developmental capacity of mouse oocytes cryopreserved before and after maturation in vitro. *J. Reprod. Fertil.*, 89: 43-50.
- Snedecor, G.W. and Cochran, W.F. (1980):* Statistical methods. 7th Ed. Ames. Iowa State University Press.
- Totey, S.M.; Pawshe, C.H. and Singh, G. (1993):* In vitro maturation and fertilization of buffalo oocytes (*Bubalus bubalis*): Effect of media, hormones and sera. *Theriogenology*, 39: 1153 -71.
- Wu, B.; Tong, J. and Leibo, S.P. (1998):* Effect of chilling bovine germinal vesicle stages oocytes on formation of microtubules and the mitotic spindle. *Theriogenology*, 49: 1:177 (abstract).