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# *In vitro* Fertilization of Vitrified-Warmed Oocytes by Frozen Thawed Semen of Breeding Buffalo Bulls

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**Abstract.-** Vitrified-warmed oocytes are now considered to be a regular source of supply for *in vitro* fertilization (IVF) technique and therefore present study was undertaken to compare the best of several combinations of dimethyl sulfoxide (DMSO) and 1,2-propanediol (PROH) for the vitrification of buffalo oocytes. Dimethyl sulfoxide (DMSO) and PROH at a concentration of 6 M were used as cryoprotectant agents (CPAs) to vitrify oocytes at germinal vesicle stage (GV) or *in vitro* matured oocytes at metaphase II stage (M II). *In vitro* fertilization rate (cleavage rate and cleavage index) was compared to examine the treatment effect in experiment I. Frozen-thawed semen of four artificial insemination donor bulls was used to compare IVF rate of vitrified-warmed oocytes in experiment II. Overall oocyte surviving after vitrified-warming procedures was 89.6%. Cleavage rate and cleavage index of GV stage oocytes vitrified in PROH did not differ ($P>0.05$) from those of fresh oocytes (control). The GV oocytes vitrified in DMSO or M-II oocytes vitrified in PROH and DMSO showed significantly less ($P<0.05$) cleavage rate and cleavage index as compared with fresh oocytes. Therefore, GV stage oocytes vitrified in PROH were further used to check the post-thawed viability of buffalo bull semen on the basis of IVF rate. Cleavage rate was better with frozen-thawed semen of bulls number 01 and 24 ($P<0.05$) with no difference in the cleavage index among four bulls. In conclusion, vitrification of GV stage oocytes in 6 M 1,2-propanediol was more effective. The vitrified-warmed oocytes with this method were successfully used to check the viability of buffalo bull semen by IVF technique.

**Key words:** Vitrification, DMSO, propanediol, germinal vesicle, metaphase II.

## INTRODUCTION

Application of cryopreserved oocytes and embryos for assisted reproductive technology (ART) in buffalo, inspired researchers to apply freezing protocols for buffalo oocytes/embryos already developed for the cattle (Singh *et al.*, 2009). Freezing of immature and *in vitro* matured buffalo oocytes have demonstrated that vitrification is more effective for the cryopreservation of buffalo oocytes than slow freezing (Gautam *et al.*, 2008). Further studies proved that the meiotic stage affects survival rates of buffalo cumulus–oocyte complexes (COC) submitted to vitrification/warming (Sharma and Loganathasamy, 2007). Concentration of cryoprotectant agent (CPA), type and time of exposure after warming have been investigated for vitrification of oocytes. Gautam *et al.* (2008) reported higher percentage of morphologically normal oocytes recovered after vitrification in dimethyl sulfoxide (DMSO) and 1,2-propanediol (PROH). Improved efficiency was achieved by employing 20% DMSO and 20% ethylene glycol by using a two-step equilibration and supplementation with 0.5 M sucrose in the final solution during vitrification and a multi-step warming procedure, starting from concentrated sucrose solution (Attanasio *et al.*, 2009). Production of blastocysts from immature buffalo oocytes vitrified in traditional French straws was reported, although the efficiency remained low (Wani *et al*., 2004).

The emergence of IVF technology has opened the way to some new approaches to evaluate bull fertility and evaluation of semen (Larsson and Rodriguez-Martinez, 2000). During IVF, spermatozoa have to mimic the functional changes that occur during *in vivo* gamete's interaction (Graham and Mocé, 2005). Thus IVF reflects the multiple functions of spermatozoa such as viability, motility, morphology, acrosome status and the ability to penetrate the oocyte investments and has diagnostic significance.

In order to optimize conditions for the vitrification of buffalo oocytes, stage of oocytes maturation and type of CPA was investigated in the present study. However, the ultimate objective was the application of vitrified oocytes to test the viability of frozen-thawed buffalo bull semen.

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## MATERIALS AND METHODS

All chemicals were reagent grade (Sigma–Aldrich Chemie GmbH, Germany) or otherwise indicated.

*Oocyte collection,* in vitro *maturation and* in vitro *fertilization*

Method of COC recovery, IVM and IFV was the same as reported earlier for buffalo (Mehmood *et al.*, 2007). Briefly, COCs were recovered from slaughter house buffalo ovaries by aspiration and *in vitro* matured in medium 199 with 10% oestrus buffalo serum (OBS). Semen from 4 breeding buffalo bulls (maintained at our centre) was cryopreserved as detailed earlier (Rasul *et al*., 2000) and further used for IVF. Sperm pellet separated with swim-up was diluted 1:1 with 100 µg/ml heparin solution in TALP and incubated for 30 min for capacitation. Sperm dose used for IVF was 1–$2\times10^6$ sperm/ml. After 48 hrs of co-incubation, eggs were washed with medium 199 by vigorous pipetting and examined under stereomicroscope to evaluate cleavage rate (number of oocytes cleaved×100/total COCs incubated) and cleavage index (number of oocytes > two-cell stage×100/number of oocytes cleaved).

*Vitrification and warming*

Vitrification was done following the method of Wani *et al*. (2004) with a modification. TCM 199 was used as basic vitrification solution instead of Dulbecco's phosphate buffered saline (DPBS). Three solutions of 6, 3 and 1.5 M DMSO or PROH were prepared in TCM 199 as basic vitrification solution containing 0.5 M sucrose and 0.4 % BSA. The oocytes were exposed to 1.5 M solution for 5 min, 3 M solution for 2 min and finally exposed to 6 M CPA solution for 30–40 sec. The oocytes were loaded in 0.25 mL straws (5–10 oocytes/straw) in the middle column of the vitrifying solution separated by air bubbles from 40 µL of the same medium on each side. Straw were sealed with polyvinyl chloride (PVC) powder and immediately dipped vertically in liquid nitrogen. All the procedures from the exposure of COCs to the final vitrification solution to dipping of the loaded straws into liquid nitrogen were done within 40 sec. Each straw was stored in liquid nitrogen for 7 days.

The straws were warmed in air (20°C) for 30 sec. The vitrified-warmed oocytes were immediately expelled in a Petri dish containing 2 mL of 0.5 M sucrose solution in TCM 199 and kept for 5 min. The oocytes were then sequentially transferred to 0.25 and 0.1 M sucrose in TCM 199 each for 5 min. The morphological appearance of oocytes after warming was evaluated with a stereomicroscope at 250 x. Those oocytes with spherical and symmetrical shape exhibiting no signs of shrinkage of ooplasm or zona cracking were considered as having survived. These oocytes were washed three times in M 199 with 10% OBS and further processed for IVM and IVF.

*Experimental design and statistical analysis*

In experiment I oocytes were randomly divided into five groups *i.e.,* 2 cryoprotectant agents (dimethyl sulfoxide and 1,2-propanediol) each used to vitrify immature oocytes at germinal vesicle (GV) stage or *in vitro* matured oocytes at metaphase II (M II) using non-vitrified oocytes as control. *In vitro* fertilization was conducted with pooled semen of 4 buffalo bulls. In experiment II oocytes vitrified with propanediol at GV stage were used to compare the post-thaw viability of buffalo semen on the basis of IVF rate. Semen from 4 buffalo bulls was collected and frozen at a weekly interval for three weeks. Frozen-thawed semen of each bull was pooled (1 straw/week) and further used for IVF. The experiments were repeated three times (replicate).

Cleavage rate and cleavage index were compared by Chi-square analysis using Minitab 12.22 (Minitab 12.22, 1996).

## RESULTS

Two CPAs, DMSO and PROH were compared to vitrify buffalo oocytes at GV or M II stages in experiment I (Table I). After warming 89.6% (328 out of 366 vitrified oocytes of three replicates) were morphologically judged as normal. Cleavage rate and cleavage index of oocytes vitrified in PROH at GV stage (PROH-GV) was 70.0% and 55.6%, respectively and did not differ from those of fresh oocytes (control) where cleavage rate and cleavage index was 73.6% and

61.7%, respectively. The oocytes from other three treatment groups showed significantly lower (P<0.05) cleavage rate and cleavage index than those of fresh oocytes (control).

**Table I.- Effect of cryoprotectant agent and stage of oocyte maturation on *in vitro* fertilization (cleavage rate and cleavage index) of vitrified-warmed oocytes**

| Treatment group | Cleavage rate (frequency) | Cleavage index (frequency) |
|---|---|---|
| Control | 73.6[a] (81/110) | 61.7[a] (50/81) |
| DMSO-GV | 46.8[b] (36/77) | 36.1[b] (13/36) |
| DMSO-MII | 49.3[b] (37/75) | 37.8[b] (14/37) |
| PROH-GV | 70.0[a] (63/90) | 55.6[a] (35/63) |
| PROH-MII | 44.2[b] (38/86) | 31.6[b] (12/38) |

Values in a column with different superscript differ (P<0.05)
Cumulative data of 3 trials (replicate)
Control, freshly collected oocytes; DMSO-GV, Germinal vesicle stage oocytes vitrified in dimethyl sulfoxide; DMSO-MII, Metaphase II stage oocytes vitrified in dimethyl sulfoxide; PROH-GV, Germinal vesicle stage oocytes vitrified in1,2-propanediol; DMSO-MII, Metaphase II stage oocytes vitrified in1,2-propanediol

Oocytes at GV stage vitrified with PROH (PROH-GV) showed the best treatment combination and were further used to compare the IVF rate of 4 buffalo bulls (Table II). Cleavage rate of buffalo bull No. 24 and 01 was 72.2% and 66.3% respectively and was significantly better (P<0.05) than the cleavage rate of buffalo bull No. 20 (45.9%) and 02 (41.1%). Cleavage index of 4 buffalo bull ranged 29.7-55.4% with non-significant difference.

## DISCUSSION

Present study was envisaged to overcome the problem of adequate supply of oocytes for *in vitro* production of embryos as the number of oocyte recovered from slaughter house buffalo ovaries was quite low (Nandi *et al.*, 2002). It has been reported that vitrification is an effective technique for the cryopreservation of buffalo oocytes than slow freezing rate (Gautam *et al*., 2008). Cleavage rate, as measured at 48 hrs after IVF, reflects the viability of the oocytes and the blastocyst rate reflects the ability of the zygote to develop for 5-7 days post-insemination (Merkies *et al.*, 2000). Therefore, a simpler method of oocytes vitrification in a straw and assessment of their viability by cleavage rate and cleavage index was the method of choice in this study. An overall of 89.6% were morphologically judged as normal after warming in the present study. The different types of damage observed were cracked zona pellucida and shrunken/fragmented ooplasm as reported earlier (Gautam *et al.*, 2008). The survival rate in the present study was comparable to the findings in bovine oocytes after solid-surface vitrification (Atabay *et al.*, 2004) and open pulled straw vitrification (Vajta *et al.*, 1998). However, vitrification of oocytes vitrification in 0.25 mL was a simple method to handle as reported earlier (Dhali *et al.*, 2000).

**Table II.- Evaluation of buffalo bull semen by *in vitro* fertilization (cleavage rate and cleavage index) of vitrified-warmed oocytes.**

| Bull No. | Cleavage rate (frequency) | Cleavage index (frequency) |
|---|---|---|
| 20 | 45.9[a] (45/98) | 37.8 (17/45) |
| 01 | 66.3[b] (63/95) | 47.6 (30/63) |
| 02 | 41.1[a] (37/90) | 29.7 (11/37) |
| 24 | 72.2[b] (65/90) | 55.4 (36/65) |

Values in a column with different superscript differ (P<0.05)
Cumulative data of 3 trials (replicate).
Germinal vesicle stage oocytes vitrified were with 1,2-propanediol

DMSO and PROH were compared to vitrify buffalo oocytes at GV or M II stage. Cleavage rate and cleavage index of oocytes vitrified in PROH at GV stage (PROH-GV) was 70.0% and 55.6% respectively and did not differ (P>0.05) from those of fresh oocytes (control). Better cleavage rate of *in vitro* matured oocytes vitrified with 7 M DMSO and PROH was reported by Wani *et al.* (2004), but cleaved oocytes were 29.8% and 27.3%, respectively. The cryopreservation of mature oocytes (M II) with slow cooling (Saunders and Parks, 1999) or vitrification protocol (Albarracin *et al.*, 2005) was reported to have deleterious effect on the meiotic spindle at M II stage of cow oocytes. *In vitro* fertilization rate of vitrified-warmed buffalo oocytes at GV stage were higher in the present study because vitrification

process preserved the cytoskeleton organization of GV stage oocytes, lacking an organized meiotic spindle, as reported by Luciano *et al.* (2009).

Oocytes vitrified by PROH at GV stage (PROH-GV) showing better *in vitro* fertilization rate in experiment I was further used for testing semen quality of four semen donor buffalo bulls maintained at our research station. Semen of three ejaculates (collected at weekly intervals) was cryopreserved and pooled semen (1 straw / ejaculate) was used for IVF in order to minimize the ejaculate variation within the bull. Cleavage rate was better with frozen-warmed semen of bull 01 and 24 with no difference in the cleavage index among four bulls. To the best of our knowledge, this is the first report on the use of vitrified oocytes for *in vitro* testing of buffalo bull semen. However, a number of studies indicated that *in vitro* cleavage rate of fresh oocytes is highly correlated with *in vivo* bull fertility (Larsson and Rodriguez-Martinez, 2000; Walters *et al.*, 2004) as during IVF spermatozoa have to mimic the functional changes that occur during *in vivo* gamete's interaction (Graham and Mocé, 2005).

In the light of above stated discussion it seems quite logical to conclude that the vitrification of GV stage oocytes with 6 M PROH is more effective than vitrification of oocytes at M II stage either with DMSO or PROH. This vitrification method demonstrated the potential to check the viability buffalo bull semen by IVF technique.

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