Vitrification of immature bubaline cumulus oocyte complexes by the open-pulled straw and conventional straw methods and their subsequent *in vitro* fertilization

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ABSTRACT: The *in vitro* maturation (IVM), fertilization (IVF) and morphological changes in buffalo cumulus oocyte complexes (COCs) cryopreserved by ultra rapid freezing using conventional (CON) and open-pulled straw (OPS) methods were tested. COCs were cryopreserved using a vitrification solution comprising of DPBS + 0.5M sucrose + 0.4% BSA and two concentrations (4.5 or 5.5M) of each cryoprotectant ethylene glycol (EG) and dimethylsulfoxide (DMSO) and cryopreserved by either CON or OPS method. Vitrified COCs were stored in LN_2 for seven days and then thawed, and morphologically normal COCs were used for IVM (n = 864) and IVF (n = 933) in two separate experiments to record (1) morphological damage of COCs due to vitrification, (2) nuclear maturation 24 h after culture (nine replicates) and (3) fertilization 24 h after insemination (10 replicates). The COCs were matured *in vitro* in TCM-199 medium using hormone supplements and fertilized using TALP-BSA. Freshly collected COCs were separately used for IVM (n = 110) and IVF (n = 130) and kept as control. The arcsin transformed data of the proportion of COCs matured or fertilized was compared by DNMR test. The highest proportion of morphologically normal COCs were seen in 5.5M EG with CON method (94.5%) and the lowest were seen in 4.5M DMSO with OPS method (82.4%). At the end of Experiment 1, it was revealed that IVM in all vitrification groups was significantly lower ($P < 0.05$) compared to control (66.4%). Amongst the various vitrification treatments the highest IVM was seen in 5.5M EG with OPS method (39.2%) and the lowest in 4.5M DMSO with CON method (19.3%). Comparison of both concentrations of EG and DMSO showed that the proportion of COCs attaining metaphase-II (M-II) increased with increasing concentration of both the cryoprotectants. However, at equal concentration of EG and DMSO the proportion of COCs attaining M-II were significantly higher in OPS method compared to CON method. In Experiment 2, a significantly higher ($P < 0.05$) IVF was seen for fresh COCs (45.4%) compared to vitrified COCs. Amongst the vitrification treatments the highest fertilization was seen for 5.5M EG with the OPS method (33.6 %) and the lowest for the 4.5M DMSO with CON method (15.17%). A dose dependant increase in the proportion of oocytes fertilized was seen with increasing concentration of both EG and DMSO [CON: 4.5M (15.2%), 5.5M (25.6%), OPS: 4.5M (21.3%) and 5.5M (27.5%)] in both CON and OPS methods. Comparison of the two cryoprotectants revealed that EG was better compared to DMSO. At equal concentrations of EG or DMSO a significantly higher ($P < 0.05$) proportion of fertilized oocytes were seen in OPS method compared to the CON method. It was concluded that developmental capacity of vitrified buffalo COCs could be improved by using OPS in comparison to conventional straws.

Keywords: buffalo; *in vitro* fertilization (IVF); oocytes; open-pulled straw (OPS); vitrification

Controlled slow freezing continues to be the most widely used technique for cryopreservation of oocytes and *in vivo* and *in vitro* produced embryos. However, in the last decade the vitrification technique, a cryoprotectant system involving the addition of higher concentrations of cryoprotectants

and ultra rapid cooling (Rall and Fahy, 1985) has been tested in various species with good results (Massip et al., 1986; Vajta et al., 1998; Dattena et al., 2000; Isachenko et al., 2003; Garg and Purohit, 2007). Different vitrification protocols have been experimented to cryopreserve *in vivo* and *in vitro* produced embryos or oocytes. These protocols differ in many ways, including the type and concentration of the cryoprotectant, number of equilibration steps, type of cryopreservation device used, time of exposure, and number of dilution steps at warming (Szell et al., 1990; Schieve, 1991; Yang et al., 1992; Ali and Shelton, 1993; Vajta et al., 1998; Dattena et al., 2000), and, it is widely believed that each of these factors can affect the results.

Glass ampoules were originally used to store frozen cattle embryos (Massip et al., 1987). In the 1980's, the plastic straw became the container of choice for vitrification and storage of embryos (Rall and Fahy, 1985; Massip et al., 1989). A recent modification, the open-pulled straw (OPS), reduces the volume of vitrification solution and allows direct contact between the embryo/oocyte and liquid nitrogen, thus enhancing the cooling rate (Vajta et al., 1998; Dattena et al., 2000; Isachenko et al., 2003). In fact, the minimum drop size and a rapid cooling rate are very important factors to improve vitrification results (Martino et al., 1996a; Arav and Zeron, 1997; Vajta et al., 1998).

Relatively few studies have focused on the cryopreservation of bubaline oocytes (Dhali et al., 2000a; Wani et al., 2004a, b; Yadav et al., 2008) and embryos (Misra et al., 1992). Buffalo oocyte cryopreservation can be important because of the relatively lower oocyte yield per ovary (Purohit and Sharma, 2002; Purohit et al., 2003) however, the cryopreservation protocols need substantial improvement. The objective of the present study was to vitrify bubaline oocytes using conventional straw and OPS methods with two cryoprotectants in order to determine the optimum cryoprotectant and vitrification method.

MATERIAL AND METHODS

Oocyte collection

Cumulus oocyte complexes (COCs) (n = 2 752) were collected by aspiration of non-attretic surface follicles over buffalo ovaries procured from a local abattoir in tissue culture medium-199 (TCM-199) buffered with 25mM Hepes and supplemented with 0.25mM pyruvate and antibiotics. The COCs with an intact compact cumulus and homogeneous ooplasm (n = 2 250) were randomly allocated to two ultra-rapid cryopreservation protocols, the conventional vitrification (CON) or vitrification using open-pulled straws (OPS) using two concentration each (4.5M and 5.5M) of the two cryoprotectants dimethylsulfoxide (DMSO) and ethylene glycol (EG).

Vitrification procedures

The basic vitrification solution (VS) was prepared by adding either, 4.5M or 5.5M concentration of either DMSO or EG to dulbecco's phosphate buffered saline (DPBS) with 0.4% bovine serum albumin (BSA) and 0.5M sucrose. The 50% VS was prepared by dilution of VS in DPBS. The open-pulled straws were made as per previously described methods (Hurtt et al., 2000). Briefly, conventional 0.25 ml French medium semen straws (IMV, France) were held horizontally 3–5 cm above a kitchen hot plate and warmed to 200°C to 250°C for 10 to 15 s or until the straw became soft. When slightly melted, the straw was pulled to approximately twice its original 13 cm length, resulting in halving of the inner diameter. After pulling, the straw was held for 10 s away from the hot plate until it solidified. Straws were then cut, with a sharp razor blade on a hard surface at 9.5 cm from the remaining straw, resulting in preparation of two open pulled straws. The straws were placed in 70% ethanol overnight and allowed to dry in a clean cabinet in a plastic container.

The oocytes to be used for cryopreservation were pre-equilibrated in 50% of the VS for 3–5 min and then placed in VS. In conventional method of cryopreservation (Yadav et al., 2008) the oocytes were loaded in 0.25 ml mini semen straws in a column of VS separated by air bubbles on each side and after loading the straws were heat sealed. The straws were pre-cooled by keeping the straw over LN_2 vapor for 2 min at the height of about 5 cm from the LN_2 level. The straws were then plunged in LN_2 for storage. In OPS system, the loading of oocytes was done by touching the tip of open pulled straw in the VS micro drop and the open-pulled straws were directly plunged into LN_2 without sealing the open ends. The straws were cryopreserved and kept for seven days before evaluation. At warming straws were placed for 10 s in water at 37°C.

Morphological evaluation

The COCs were defined as having morphologically survived if the cells possessed an intact zona pellucida and plasma membrane and homogeneous cytoplasm. The number of morphologically normal COCs was recorded for each replicate and each treatment separately. In two separate experiments, morphologically normal COCs were further subjected to *in vitro* maturation (n = 864) and fertilization (n = 933) as per previously described methods (Purohit et al., 2005). Freshly collected COCs were separately used for IVM (n = 110) and IVF (n = 130) and kept as control.

In vitro maturation

In Experiment 1 (nine replicates) warmed COCs were separately cultured *in vitro* in TCM-199 medium supplemented with 5 µg/ml FSH, 5 µg/ml LH, 1 ng/ml estradiol, 25mM Hepes, 0.25mM pyruvate and antibiotics in 50–100 µl maturation media for 24 h at 38.5 ± 1°C and 5% CO_2 in humidified air in a CO_2 incubator. After 24 h COCs were taken out, and the surrounding cumulus cells were separated by vortexing for 1 min in TCM-199 with hyaluronidase (0.3%). They were then fixed and stained with 1% orcein to evaluate the nuclear status as per previously described methods (Purohit et al., 2005).

In vitro fertilization

In Experiment 2, (10 replicates) vitrified and warmed COCs were first matured *in vitro* for 24 h and then subjected to *in vitro* fertilization (IVF). Frozen thawed buffalo bull semen was prepared for IVF using a discontinuous Percoll density gradient to separate live spermatozoa (Purohit et al., 2005). Briefly, 4 ml of 90% isotonic Percoll was layered in a 15 ml centrifuge tube beneath 4 ml of 45% isotonic Percoll. The sperms were washed initially in TALP-BSA by centrifugation at 250g for 4 min. The sperm pellet was re-suspended in 1 ml of the medium. The washed sperm pellet was layered on the top of Percoll gradient, and centrifuged at 300g for 35 min. The resultant pellet was removed from the bottom and washed twice in TALP-BSA by centrifugation.

The sperm pellet was then re-suspended in TALP-BSA to give a final concentration of 3×10^9 sperms per ml. They were then incubated for 2–3 h in a CO_2 incubator. The matured COCs were transferred to another dish containing Fert-TALP medium (TALP supplemented with 30 mg penicillamine per ml, 15mM hypotaurine per ml) under paraffin oil. This was inseminated with prepared sperms in a volume so as to give a final concentration of 2×10^5 sperms per ml.

Following co-incubation for 20–24 h with sperms, all COCs were washed with fresh medium and vortexed for 1 min to separate the cumulus cells. They were then fixed and stained to evaluate fertilization.

Statistical methods

The proportion of COCs that were retrieved in morphologically normal form over the various treatments and cryopreservation protocols, were compared by chi-square test. Arcsin transformation of the proportion of oocytes matured and fertilized *in vitro* over the various treatment groups was done and the replicate data compared by Duncans new multiple range test (DNMRT).

RESULTS

Morphologic survival

In Experiment 1 and 2 the morphologic survival of oocytes was 88.79% (864/973) and 85.6% (933/1 090). The highest proportion of morphologically normal oocytes were seen in 5.5M EG with the CON method and the lowest were in 4.5M DMSO, with the OPS method for both Experiment 1 and 2. Non-significant ($P > 0.05$) differences were seen between the oocytes recovered in morphologically normal form for equal concentrations of both EG and DMSO with the two methods of vitrification used. Pooled data of morphologic survival of oocytes in the various concentrations of G and EG or their combination are shown in Table 1.

In vitro maturation

Oocyte maturation was significantly lower ($P < 0.05$) in all the vitrification cryoprotectants compared to control. The proportion of oocytes attaining M-II was significantly higher ($P < 0.05$) at 5.5M concentration compared to 4.5M concentration of both EG and DMSO in both methods of vitrifica-

Table 1. Morphologic survival of buffalo oocytes vitrified using two methods of vitrification and two concentrations of EG and DMSO

Vitrification solution	Method of vitrification[A]	Proportion of oocytes seen to be morphologically normal
4.5M EG	CON	89.6^{ac}
5.5M EG	CON	94.6^{a}
4.5M DMSO	CON	86.5^{bcd}
5.5M DMSO	CON	92.0^{ad}
4.5M EG	OPS	86.6^{bcd}
5.5M EG	OPS	89.4^{ac}
4.5M DMSO	OPS	82.3^{bc}
5.5M DMSO	OPS	88.8^{abd}

Values in same column with different superscripts differ significantly, X^2 test ($P < 0.05$)
[A]pooled data of two experiments
CON = conventional method; OPS = open-pulled straw method

tion used. The highest IVM was seen at 5.5M EG using the OPS method and the lowest was seen in 4.5M DMSO with the CON method. At equal concentrations of EG and DMSO the proportion of oocytes attaining M-II was significantly higher in the OPS method compared to the CON method and EG was better compared to DMSO.

In vitro fertilization

In Experiment 2, a significantly higher ($P < 0.05$) IVF was seen for fresh COCs (45.4%) compared to vitrified COCs. Amongst the vitrification treatments the highest fertilization was seen for 5.5M EG with the OPS method (33.6%) and the lowest for the 4.5M DMSO with CON method (15.17%). At a higher concentration of both EG and DMSO (5.5M) an increase in the proportion of oocytes fertilized was seen in both CON and OPS methods. Comparison of the two cryoprotectants revealed that EG was better compared to DMSO. At equal concentrations of EG or DMSO a significantly higher ($P < 0.05$) proportion of fertilized oocytes were seen in OPS method compared to the CON method. It was concluded that developmental capacity of

Table 2. *In vitro* maturation and fertilization of buffalo oocytes vitrified by two methods in different concentrations of EG and DMSO

Vitrification solution	Vtrification method	*In vitro* maturation (%)[A]	*In vitro* fertilization (%)[B]
Control		63.36^{a}	45.38^{a}
4.5M EG	CON	23.91^{e}	18.64^{d}
5.5M EG	CON	31.37^{c}	28.09^{b}
4.5M DMSO	CON	19.31^{f}	15.17^{e}
5.5M DMSO	CON	29.78^{cd}	25.64^{b}
4.5M EG	OPS	29.41^{cd}	24.77^{bc}
5.5M EG	OPS	39.13^{b}	33.61^{f}
4.5M DMSO	OPS	25.55^{de}	21.36^{cd}
5.5M DMSO	OPS	36.04^{b}	27.50^{b}

Values in same column with different superscripts differ significantly, DNMR-test (arcsin transformed data) ($P < 0.05$)
CON = conventional method; OPS = open-pulled straw
[A]data pooled from 9 replicates; [B]data pooled from 10 replicates

vitrified buffalo COCs could be improved by using OPS in comparison to conventional straws.

DISCUSSION

The results of the present study indicated that a high proportion of COCs retain their morphology after a short exposure to high concentration of different cryoprotectants and cryopreservation with different vitrification protocols. Previous studiees on vitrified cattle oocytes (Vieira et al., 2001; Mavrides and Moroll, 2002; Yang-Byoung et al., 2003) had recorded the morphological survival rates of between 65 to 95% and, studies on buffalo oocytes had also recorded high proportion of oocytes in morphologically normal form subsequent to vitrification in either DMSO or EG (Dhali et al., 2000a; Wani et al., 2004b; Yadav et al., 2008). As compared to conventional method of vitrification (CON) the OPS method yielded lower proportion of morphologic survival of oocytes as also shown in previous studies on mouse oocytes (Shee-Uan-Chen et al., 2000). A portion of the oocytes in the OPS might come into direct contact with liquid nitrogen which may have an adverse effect on their survival.

The *in vitro* maturation and fertilization of vitrified immature buffalo COCs was significantly lower compared to non-vitrified control in the present study. Since the production of the first blastocyst stage embryos following IVF of frozen-thawed mature bovine oocytes (Lim et al., 1991) many research teams have tried to improve cryopreservation procedures for bovine oocytes. Despite the efforts, survival of cryopreserved oocytes and subsequent blastocyst development remained low (Fuku et al., 1992; Otoi et al., 1993; Suzuki et al., 1996; Kubota et al., 1998). The reduction in the developmental ability of vitrified oocytes could be due to the toxic effects of cryoprotectants and osmotic injury. In addition, the possibility of deleterious effects on chromosomes and other cytoplasmic structures cannot be ruled out, since such effects have been shown during cryopreservation of mouse (Van der Elst et al., 1988) and human (Pickering et al., 1990; Park et al., 1997) oocytes.

The freeze thaw process is known to induce an alteration in the physico-chemical properties in the intra-cellular lipids (Isachenko et al., 2001; Kim et al., 2001) and such damages may render the oocyte incapable of retaining its developmental competence. Despite the protective effects of cryoprotectants during freezing, they may impose concentration, time and temperature dependent toxicity (Fahy et al., 1990).

The 5.5M concentration of EG was found to be a better cryoprotectant compared to 4.5M concentration for both methods (CON and OPS) of vitrification of oocytes as evident by higher proportion of oocytes maturing or fertilizing *in vitro* (Table 2). Moreover, EG was found to be a better cryoprotectant compared to DMSO in all the treatments, and cryopreservation protocols tested, as shown by a higher proportion of vitrified warmed oocytes maturing or fertilizing subsequently. Vitrified warmed buffalo oocytes have been shown to yield highest subsequent *in vitro* maturation and fertilization, when they had been vitrified in 6M (Yadav et al., 2008) to 7M solutions of various cryoprotectants compared to that in lower concentrations, and that EG was better compared to DMSO (Wani et al., 2004b). EG is known to offer advantage over other cryoprotectants in terms of higher permeation into oocytes for vitrification and, faster removal during dilution as its molecular weight is lower (Dhali et al., 2000b).

The subsequent *in vitro* maturation and *in vitro* fertilization rates of vitrified buffalo oocytes were higher for oocytes vitrified by the OPS method, compared to the CON method at same concentrations of both the cryoprotectants. This confirms the findings of Vajta et al., (1998) that the developmental capacity of vitrified bovine oocytes could be improved using the OPS in comparison to the CON method. The tip of the open-pulled straw is designed to have a small diameter and thin wall, hence, oocytes held in OPS with a very small volume of vitrification solution achieve a faster cooling and warming rate (a theoretical rate of 20 000°C/min) than those in conventional straw (2 500°C/min) (Rall and Fahy, 1985; Vajta et al., 1998). They can rapidly traverse the critical temperature damaging to the spindle and assumed to be 15°C to –15°C (Martino et al., 1996b). Moreover, oocytes in vitrification medium (1–2 µl) in OPS are directly warmed in the dilution and quickly expelled (within 1 s) and immediately diluted. This reduces exposure to inappropriate temperatures and concentrate cryoprotectants. In contrast, the conventional straw is warmed in water and then cut with scissors. It takes more time for oocytes to pass through the unsuitable conditions (< 45 s). These effects may explain why vitrification of oocytes using OPS results in better *in vitro* maturation and fertilization.

In conclusion the developmental capacity of vitrified buffalo oocytes could be improved by using OPS in comparison to the conventional straw method.

REFERENCES

Ali J., Shelton J.N. (1993): Vitrification of preimplantation stages of mouse embryos. Journal of Reproduction and Fertility, 98, 459–465.

Arav A., Zeron Y. (1997): Vitrification of bovine oocytes using modified minimum drop size technique (MDS) is effected by the composition and concentration of the vitrification solutions and by the cooling conditions. Theriogenology, 47, 341.

Dattena M., Ptak G., Loi P., Cappai P. (2000): Survival and viability of vitrified *in vitro* and *in vivo* produced ovine blastocysts. Theriogenology, 51, 1511–1519.

Dhali A., Manik R.S., Das S.K., Singla S.K., Palta P. (2000a): Vitrification of buffalo (*Bubalus bubalis*) oocytes. Theriogenology, 53, 1295–1303.

Dhali A., Manik R.S., Das S.K., Singla S.K., Palta P. (2000b): Post vitrification survival and *in vitro* maturation rate of buffalo (*Bubalus bubalis*) oocytes: effect of ethylene glycol concentration and exposure time. Animal Reproduction Science, 63, 159–165.

Fahy G.M., Lilley T.H., Linsdell H., Douglas M.S., Meryman H.T. (1990): Cryoprotectant toxicity and cryoproectant toxicity reduction in search of molecular mechanism. Cryobiology, 27, 247–268.

Fuku E.T., Kojima Y., Shioya G.J., Marcus S., Downey B.R. (1992): *In vitro* fertilization and development of frozen thawed bovine oocytes. Cryobiology, 29, 485–492.

Garg N., Purohit G.N. (2007): Effect of different cryoprotectant concentrations for ultrarapid freezing of immature goat follicular oocytes on their subsequent maturation and fertilization *in vitro*. Animal Reproduction, 4, 113–118.

Hurtt A.E., Landim A.F., Seidel G.E., Squires E.L. Jr. (2000): Vitrification of immature and mature equine and bovine oocytes in an ethylene glycol, ficoll and sucrose solution using open-pulled straws. Theriogenology, 54, 119–128.

Isachenko V., Michelman H.W., Alabart J.L., Vazquez I., Isachenko E., Bezugly N., Nawroth F. (2001): Lipolysis and ultrastructural changes of intra cellular lipid vesicles after cooling of bovine and porcine GV oocytes. Anatomia Histologia Embryologica, 30, 333–338.

Isachenko V., Folch J., Isachenko E., Nawroth F., Krivokharchenko A., Vajta G., Dattena M., Alabart J.L. (2003): Double vitrification of rat embryos at different developmental stages using an identical protocol. Theriogenology, 60, 445–452.

Kim J.Y., Kinoshita M., Ohnishi M., Fukui, Y. (2001): Lipid and fatty acid analysis of fresh and frozen-thawed immature and in vitro matured bovine oocytes. Reproduction Cambridge, 122, 131–138.

Kubota C., Yang S., Dinneyes A., Todoroki J., Yamakushi H., Mizoshita K., Imohae S., Tabara N. (1998): *In vitro* and *in vivo* survival of frozen thawed bovine oocytes after IVF, nuclear transfer and parthenogenetic activation. Molecular Reproduction and Development, 51, 281–286.

Lim J.M., Fuku Y., Ono H. (1991): The post thaw developmental capacity of frozen bovine oocytes, following in vitro maturation and fertilization. Theriogenology, 35, 1225–1235.

Martino A., Polland J.W., Leibo S.P. (1996a): Effect of chilling bovine oocytes on their developmental competence. Molecular Reproduction and Development, 45, 503–512.

Martino A., Songsasen N., Leibo S.P. (1996b): Development into blastocysts of bovine oocytes cryopreserved by ultra rapid cooling. Biology of Reproduction, 54, 1059–1069.

Massip A., Van der Zwahlen P., Scheffen B., Ectors F. (1986): Pregnancies following transfer of cattle embryos preserved by vitrification. Cryo-Letters, 7, 270–273.

Massip A., Van der Zwalmen P., Ectors F. (1987): Recent progress in cryopreservation of cattle embryos. Theriogenology, 27, 69–79.

Massip A., Van der Zwahlen P., Scheffen B., Ectors F. (1989): Some significant steps in the cryopreservation of mammalian embryos with a note on a vitrification procedure. Animal Reproduction Science, 19, 117–129.

Mavrides A., Morroll D. (2002): Cryopreservation of bovine oocytes: Is cryoloop vitrification the future to preserving the female gamete? Reproduction Nutrition and Development, 42, 73–80.

Misra A.K., Joshi B.V., Agrawala P.L., Kasiraj R., Sivaiah S., Rangareddi N.S., Siddiqui M.V. (1992): Cryopreservation of bubaline embryos. Buffalo Journal, 8, 297–303.

Otoi T., Tachikawa S., Sondo S., Suzuki T. (1993): Developmental capacity of bovine oocytes frozen in different cryoprotectants. Theriogenology, 40, 801–807.

Park S., Son W., Le S., Lee K.J., Cha K. (1997): Chromosomes and spindle configuration of human oocytes matured *in vitro* after cryopreservation at germinal vesicle stage. Fertility and Sterility, 68, 920–926.

Pickering S.J., Barode P.R., Johnson M.H., Cant A., Currie A., Currie J. (1990): Transient cooling to room

temperature can cause irreversible disruption of the meiotic spindle in the human oocyte. Fertility and Sterility, 54, 102–108.

Purohit G.N., Sharma S.S. (2002): *In vitro* maturation of bubaline oocytes in serum free media in the *in vivo* bovine vagina. Veterinary Record, 151, 640–641.

Purohit G.N., Duggal G.P., Dadarwal D., Kumar D., Yadav R.C., Vyas S. (2003): Reproductive biotechnologies for improvement of buffalo: The current status. Asian Australasian Journal of Animal Science, 16, 1071–1086.

Purohit G.N., Brady M.S., Sharma S.S. (2005): Influence of epidermal growth factor and insulin like growth factor-1 on nuclear maturation and fertilization of buffalo cumulus oocyte complexes in serum free media and their subsequent development *in vitro*. Animal Reproduction Science, 87, 229–239.

Rall W.F., Fahy G.M. (1985): Ice-free cryopreservation of mouse embryos at –196 degrees C by vitrification. Nature, 313, 573–575.

Schieve M.C. (1991): The science and significance of embryo cryopreservation. Journal of Zoo and Wildlife Medicine, 22, 6–22.

Shee-Uan-Chen C., Yie-Ron L., Hsin-Fu C., Kuang-Han C., Hong-Nerng H., Yu-Shih Y. (2000): Open-pulled straws for vitrification of mature mouse oocytes , preserve patterns of meiotic spindle and chromosomes better than conventional straws. Human Reproduction, 15, 2598–2603.

Suzuki T., Boediono A., Takagi M., Saha S., Sumentra I.C. (1996): Fertilization and development of frozen-thawed germinal vesicle bovine oocytes by one-step dilution method *in vitro*. Cryobiology, 33, 515–524.

Szell A., Zhang J., Hudson R. (1990): Rapid cryopreservation of sheep embryos by direct transfer into liquid nitrogen vapor at –180 degrees C. Reproduction Fertility and Development, 2, 613–618.

Vajta G., Holm P., Kuwayama M., Booth P.J., Jacobson H., Greve T., Callesen H. (1998): Open pulled straws (OPS) vitrification : a new way to reduce cryoinjuries of bovine oocytes and embryos. Molecular Reproduction and Development, 51, 53–58.

Van der Elst J., Van den Abdeel E., Jacobs R., Wisse E., Van Steirteghem A.C. (1988): Effect of 1,2-propanediol and dimethyl-sulfoxide on the meiotic spindle of the mouse oocyte. Human Reproduction, 3, 960–967.

Vieira D.G.I., Fontes R.S., Dias A.B., Paes C.S., Adona P.R., Bussiere M.C.C., Leal C.L.V., Pinheiro L.P.L. (2001): *In vitro* vitrification of mature bovine oocytes. XIV Congresso Brasileiro de Reproducao Animal Belo Horizonte, Brazil, 1–5 August, 2001. Revista-Brasileira de Reproducao Animal, 25, 416–417.

Wani N.A., Misra A.K., Maurya S.N. (2004a): Maturation rates of vitrified thawed immature buffalo (*Bubalus bubalis*) oocytes: Effect of different types of cryoprotectants. Animal Reproduction Science, 84, 327–335.

Wani N.A., Maurya S.N., Misra A.K., Saxena V.B., Lakhchaura B.D. (2004b): Effect of cryoprotectants and their concentration on *in vitro* development of vitrified warmed immature oocytes in buffalo (*Bubalis bubalis*). Theriogenology, 61, 831–842.

Yadav R.C.S., Sharma A., Garg N., Purohit G.N. (2008): Survival of vitrified water buffalo cumulus oocyte complexes and their subsequent development *in vitro*. Bulgarian Journal of Veterinary Medicine, 11, 55–64.

Yang N.S., Lu K.H., Gordon I., Polge C. (1992): Vitrification of bovine blastocysts produced *in vitro*. Theriogenology, 37, 326. (Abstr.)

Yang-Byoung C., Im G.S., Chang W.K., Won-Kyong Lee Y.K., Yun-Keun Oh S.J., Jin D.I., Dong L.L., Im K.S., Chang-Kyu Yang B.C., Chang W.K., Lee C.K. (2003): Survival and *in vitro* development of immature bovine oocytes cryopreserved by vitrification. Asian Australasian Journal of Animal Science, 16, 23–28.

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