Cryotop vitrification for in vitro produced bovine and buffalo (*Bubalus bubalis*) embryos at different stages of development

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Abstract: The aim of this study was to evaluate the possibility to vitrify in vitro produced (IVP) buffalo and bovine embryos at different stages of development by an advanced version of the “minimal volume approaches”: the Cryotop method. In both experiments, the embryos were vitrified at the tight morula (TM), early blastocyst (eBl), blastocyst (Bl), expanded blastocyst (xBl) and, only for buffalos, at the hatched blastocyst (hBl) stage. After warming, the embryos were cultured in vitro for 24 hours. Stage of development affected the freezability of IVP embryos of both species with the highest embryo survival rates at advanced stages (xBl=76% and hBl=75% for buffalos and xBl=75% for bovine). These results suggest that Cryotop vitrification is an efficient method for buffalo and bovine IVP embryo cryopreservation.

Key words: Vitrification, Cryotop, Buffalo, Bovine.

INTRODUCTION - In buffalo the in vitro embryo production (IVEP) technology represents the best tool to speed up the propagation of superior germplasm through the maternal lineage, due to the low efficiency of MOET programs in this species (Zicarelli, 2001). However, the diffusion in the field of IVEP technology in buffalo species is strongly dependent on the improvement of embryo cryopreservation efficiency. This technology, by allowing the preservation of embryos for an unlimited period of time, is a valid tool for an efficient application of embryo transfer program in this species overcoming the limitation due to the low numbers of available recipients and costs of breeding programs. In the last twenty years, vitrification has been considered an alternative method to cryopreserve those biological specimens that are particularly sensitive to chilling injury. In vitro produced (IVP) buffalo and bovine embryos have been previously vitrified with different methods (Vajta, 1998; Neglia, 2001; Duran, 2004; De Rosa, 2006;) with encouraging results, but the efficiency of cryopreservation still needs to be improved. Among the minimum volume vitrification methods, currently considered the most promising for vitrifying oocytes and embryos, the most recent is the Cryotop (CTV, Kuwayama *et al.*, 2005). This procedure allows to reach very high cooling (22.800°C/min) and warming (42.100°C/min) rates as a result of the extremely small volume used (<0.1µl). The CTV method has been successful to cryopreserve embryos in different species (Laowtammathron, 2005; Hiruma *et al.*, 2006). To our knowledge, the influence of the stage of development on survival of buffalo and bovine embryos after...
vitrification has not yet been evaluated. The aim of this study was to verify the possibility to vitrify IVP buffalo and bovine embryos at different stages of development by CTV.

**MATERIAL AND METHODS** - In Experiment 1, IVP buffalo embryos (N=154) of good quality were vitrified at different stages of development, reached by Day 7 of embryo culture, (TM= 25; eBl= 20; Bl= 21; xBl= 45; hBl= 44) using the CT device previously described to cryopreserve human oocytes (Kuwayama *et al.*, 2005). Embryos were equilibrated in a first solution containing 7.5 % ethylene glycol (EG) and 7.5 % dimethyl sulfoxide (DMSO), prepared in Hepes-buffered TCM199 supplemented with 20% FCS (BS), for 3 min and then transferred into a second solution containing 16.5 % EG, 16.5 % DMSO prepared in BS with 0.5 M sucrose. After 25 sec, the embryos were picked up in <0.1 µl of vitrification solution, placed on the top of a cryotop and immediately submerged into liquid nitrogen. The warming procedure was performed plunging the top of the cryotop containing the embryos into a 0.25 M sucrose solution, after 1 min the embryos were transferred into 0.15 M sucrose solution for 5 min and cultured in SOF medium for 24 hours. In Experiment 2, IVP bovine embryos (N=177) of good quality at different stages of development, reached by Day 7 of embryo culture (TM= 15; eBl= 36; Bl= 67; xBl= 59) were vitrified, warmed and cultured using the same procedures described in the experiment 1. In both experiments, embryo survival rate was determined as the percentage of vitrified-warmed embryos resuming normal morphology and re-expansion of the blastocoel cavity. The total number of embryos was obtained over 14 replicates in Experiment 1, and over 5 replicates in Experiment 2; this difference was due to the different in vitro blastocyst rate between the two species (approximately 25% for buffalo and 40% for bovine), together with the poor availability of buffalo ovaries, as source of oocytes. Differences in survival rates among the stages of development were analyzed by Chi Square test.

**RESULTS AND CONCLUSIONS** - In Experiment 1, survival rates of buffalo embryos vitrified by CTV were different among the stages of development (Figure 1). The survival rates of the xBl and hBl were significantly higher compared to the TM, eBl and Bl stages (76 and 75% vs 16, 50 and 48% with respectively P<0.01 vs TM and P<0.05 vs eBl and Bl).

Figure 1. Survival rate post-warming of buffalo embryos vitrified at different stages of development.
In cattle (Experiment 2) the survival rates were also different among the stages of development (Figure 2). The xBl stage gave a survival rate significantly (P<0.01) higher than all the other stages (75 vs 20, 42 and 51% respectively for xBl, TM, eBl and Bl). These results demonstrated that CTV is a very efficient method to cryopreserve IVP embryos at advanced stage both in bovine and buffalo species, as indicated by the higher survival rates compared to those previously reported employing the traditional straws (Neglia et al, 2001). Similar survival rates were reported for bovine and buffalo hBl cryopreserved by CTV in an earlier work in which the other stages of development were not considered (Laowtammathron et al, 2005). In our study we analyzed all the stages of development and we showed that the freezability of IVP buffalo and bovine embryos is affected by the stage; in particular, the most advanced stages (xBl and hBl, in buffalo species, and xBl in bovine) showed a higher survival rates compared to earlier ones (TM, eBl and Bl). It is worth pointing out that the endpoint of embryo culture was day 7. It follows that we can not attribute the better freezability of the advanced stages only to stage-related ultrastructural/physico-chemical properties because of their earlier development in vitro, that is synonymous of better quality of the embryos. Analogously, we speculate that the very low efficiency of the TM is, at least in part, due to the worse quality of these embryos, indicated by their delayed development in vitro.

In conclusion, it was demonstrated that CTV is a valid tool to cryopreserve IVP buffalo and bovine embryos. Furthermore, the efficient results obtained in this trial suggest that CTV may also improve the pregnancy rate following ET of cryopreserved embryos, that is, particularly in buffalo species, a major limiting factor for the diffusion of IVP technology in the field.

ACKNOWLEDGMENTS - The Cryotop device was kindly provided by Kuwayama M.

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