Viability And DNA Fragmentation Assessment Of Bovine Embryos Vitrified By Different Methods

K Gh M Mahmoud [1]*, G E Seidel Jr [2]

[1]Dept.Anim.Reprod.& A.I, National Research Center, Dokki, Tahrir Street, 12622 Giza, Egypt
[2]Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins, CO 80523-1683, USA
karima_mahmoud@hotmail.com

Abstract: This study was conducted to compare the efficiency of four vitrification methods: straws in goblet, straws in Hasler device, cryloop and cryotop methods on blastocyst viability and the extent of apoptosis using the TUNEL technique in bovine embryos. Blastocysts were produced in vitro by standard procedures. The base medium for vitrification was Syngro holding medium. Embryos were vitrified by the four methods following a two step addition of cryoprotectant. Embryos were first exposed to 5 M EG (28%) for 3 min at 20–22 °C, and then transferred into a vitrification solution composed of 6.5 M EG (36%), 0.5 M galactose (9% w/v), and 18% (w/v) Ficoll 70 in Syngro plus 1% PVA for 45 s. Embryos were loaded in the four devices and were placed in liquid nitrogen. The post-warm survival of the blastocysts was assessed by in vitro culture for 24 h. The proportions of embryos developing into expanded/hatched blastocysts were assessed after 48h. Embryos were stained and the number of blastomeres was determined by counting the number of nuclei. DNA fragmentation was assessed by TUNEL assay. No significant difference was found in survival rates directly after warming and hatching rates among the four methods. There was a significant difference ($P<0.05$) in embryo viability among the four methods at 24 h post warming. Embryos vitrified-thawed using cryotop exhibited higher proportions of development into hatched blastocysts but without any significant difference between the other methods. A significant decrease in total cell number was observed in vitrified blastocysts than fresh embryos. In vitrified expanded and hatched blastocysts, the cell number was significantly increased and mitotic cells were decreased compared to fresh embryos. All vitrification methods induce DNA damage to blastocystes. Damage was minimal in cryotop and straw in goblet and maximal with cryloop and straws in the Hasler device. In conclusion, bovine blastocyst can be successfully cryopreserved by vitrification using the four devices. Relatively low survival rates of blastocysts and increase in DNA damage were obtained using the cryoloop device compared to other methods. [Researcher. 2010;2(1):14-20]. (ISSN: 1553-9865).

Key words: TUNEL, Embryos, Vitrification, Cryloop, Cryotop.

1. Introduction

Vitrification protocols are particularly attractive cryopreservation strategies for embryos as they are inexpensive, fast, and simple, but most are embryo-toxic as the solutions contain high cryoprotectant concentrations (Kuleshova and Lopata, 2002). Considerable effort is therefore still being directed toward improvement of vitrification protocols for embryos (Mazur et al., 2008). Vitrification appears to be the method of choice for in vitro-produced embryos, which are more sensitive to cryopreservation than in vivo produced embryos (Vajta and Nagy, 2006).

Vitrification is a practical approach for embryo cryopreservation in the mouse (Kasai et al. 1990), sheep (Schiewe et al. 1991), cow (Delval et al. 1996), buffalo (Hufana-Duran et al. 2004), horse (Oberstein et al. 2001), rabbit (Hochi et al. 2004) and human (Mukaida et al. 2001). Development of vitrification procedures is characterized by ultra-rapid cooling rates. Cryoloop (Lane et al., 1999 a,b and Begin et al., 2003) and Cryotop (Kuwayama and Kato, 2000 and Mukaida et al., 2003) methods have been reported. This is in addition to methods commonly used with cattle and horses that permit "direct transfer", simply thawing/warming the plastic straw containing the embryo, shaking it (with some systems) to mix media in compartments within the straw, and then transferring the embryos directly to the uterus of a recipient without ever observing them.

Availability of embryos of high quality is required to obtain satisfactory embryonic developmental rates and normal calves following transfer of in vitro-produced bovine embryos. From a laboratory point of view, one useful measure of quality is the ability of the embryo to survive cryopreservation, as essentially all embryos from a given treatment can be tested (Lonergan et al., 2001 and Gomez et al., 2008). The presence of mitotic activity and the number of blastomeres may be the most important parameters for embryo survival. The relationship between embryo

quality and the number of blastomeres (Wurth et al., 1988) and mitotic cells (Wurth et al., 1994) in bovine embryos was investigated. Data on this topic in relation to cryoprservation are not available and are considered to be essential for the evaluation of embryo development after vitrification.

In many studies with IVMFC embryos, there is evidence of a marked deterioration in morphological quality and a much reduced viability following cryopreservation when compared with embryos recovered after superovulation (Niemann, 1991). Apoptosis is the ultimate cellular response to suboptimal conditions and different kinds of stress that an embryo might encounter during its freeze-thaw cycle. The evaluation of DNA fragmentation by terminal deoxynucleotidyl transferase (TDT)-mediated dUTP nick-end labeling (TUNEL) has been used as a reliable method for detection of apoptosis in embryos (Fahrudin et al., 2002 and Fabian et al., 2005).

The objective of the present study was to compare the effect of four methods of vitrification on viability and the extent of apoptosis using the TUNEL technique in bovine embryos.

2. Materials and methods

2.1. In vitro embryo production

Oocytes were aspirated from 3- to 8-mm follicles of abbatoir-derived ovaries from mature cull cows. Oocytes were matured in chemically defined medium (CDM) (Olson and Seidel, 2000) supplemented with 0.5% fatty acid free bovine serum albumin (FAF-BSA) (Sigma Chemical Co., St. Louis, MO, USA; A6003), 15 ng/mL NIDDK-oFSH-20, 1 mg/mL USDA-LH-B-5, 0.1 µg/mL E2, 50 ng/µL epidermal growth factor (Sigma E9644) and 0.1 mM cysteamine for 23 h at 38.5 °C and 5% CO2 in air. Sperm were separated through a Sperm-Talp (Parrish et al, 1988) Percoll (Sigma P1644) gradient and coincubated (0.5 X 10^6 sperm/mL) with matured oocytes in CDM fertilization medium supplemented with 0.5% FAF-BSA, 2 mM caffeine and 0.02% heparin (Sigma H9399) for 18 h at 38.5 °C and 5% CO2 in air. Culture was in CDM supplemented with standard nonessential amino acids (Sigma M7145) and 10 mM EDTA (CDM1) for 2.5 days, and in CDM without EDTA supplemented with essential (Sigma B6766) and nonessential amino acids (CDM2) for 4.5 days at 38.5 °C, 5% O2, 5% CO2 and 90% N2. Blastocysts were selected on day 7.

2.2. Blastocyst vitrification

All vitrification solutions were prepared in Syngro holding medium (Bioniche, Pullman, WA, and USA). Embryos were held at room temperature (20–22 °C) in 500µl Syngro until vitrification. Embryos were vitrified following a two step addition of cryoprotectant by the following four methods.

2.2.1. Vitrification in 0.25-mL straws in goblet

Blastocysts were vitrified using two-step vitrification procedure (Campos-Chillon et al., 2006). Embryos were placed in 5 M EG (28%) in Syngro plus 1% PVA for 3 min during the first step. For the second step, they were placed in 6.5 M EG (36%), 0.5 M galactose (9% w/v), and 18% (w/v) Ficoll 70 (Sigma F2778) in Syngro plus 1% PVA for 45s. Immediately, straws were placed in liquid nitrogen vapor in goblet for 1 min and then plunged into liquid nitrogen. For warming, straws were held in air for 10 s, placed in water at 37 °C for 30 s, and flicked four to six times to mix columns. After warming, embryos were washed five times in Syngro and cultured in CDM-2 plus 5 % FCS at 38.5 °C, 5% O2, 5% CO2 and 90% N2 for further 2 d.

2.2.2. Vitrification in 0.25-mL straws in Hasler device

Blastocysts were vitrified by Hasler device. It is a stainless steel cup which has a central opening the same size as the straw. The vitrification procedure is the same as nitrogen vapour except straws were placed in the opening of the container instead of in a goblet for 1 min and then plunged into liquid nitrogen.

2.2.3. Cryoloop method

The Cryoloop procedure was similar to that of Lane et al. (1999 a,b), with modifications described by Takagi et al. (2002). Briefly, blastocysts were placed in 5 M EG (28%) in Syngro plus 1% PVA for 3 min at 20–22 °C, and then transferred into a vitrification solution composed of 6.5 M EG (36%), 0.5 M galactose (9% w/v), and 18% (w/v) Ficoll 70 in Syngro plus 1% PVA for 45 s. The cryoloop consisted of a minute nylon loop (20µm wide, 0.5-0.7 mm in diameter) mounted on a stainless steel pipe inserted into the lid of a cryovial. Two blastocysts were transferred to a film of vitrification solution on a cryoloop that was previously dipped in the vitrification solution. The cryoloop was plunged into liquid nitrogen in a cryovial, and then the cryovial was screw-sealed. After storage in liquid nitrogen, embryos were thawed by immersing the loop in Syngro with

1.0 M galactose for 4 min then to 0.5M galactose for 4 min at 20–22 °C. Finally, the embryos were washed and cultured in CDM-2 plus 5 % FCS at 38.5 °C, 5% O2, 5% CO2 and 90% N2 for 2 d.

2.2.4. Cryotop method

This cryotop procedure was described by Kuwayama and Kato (2000). Briefly, blastocysts were placed in 5 M EG (28%) in Syngro plus 1% PVA for 3 min at 20–22 °C, and then transferred into a vitrification solution composed of 6.5 M EG (36%), 0.5 M galactose (9% w/v), and 18% (w/v) Ficoll 70 in Syngro plus 1% PVA for 45 s. Two blastocysts were placed to a sheet of cryotop (Kitazato Supply, Tokyo, Japan) in a small volume of the vitrification solution (<1µl). The cryotop was plunged into liquid nitrogen. After storage in liquid nitrogen, blastocysts were thawed by removing the protective cap from the cryotop under liquid nitrogen and the polypropylene strip of cryotop was immersed directly into Syngro following a stepwise dilution with 1.0 and 0.5 M sucrose solutions at 4 min intervals. Finally, the embryos were washed and cultured in CDM-2 plus 5 % FCS at 38.5 °C, 5% O2, 5% CO2 and 90% N2 for further 2 d.

2.3. Survival assay

Embryos were evaluated morphologically after thawing and after 24 h and 48 h of culture. The quality and stage of development were recorded. The embryos developed to more advanced stages, with a clearly visible inner cell mass, were considered to be survived.

2.4. Embryo staining

A total number of 25 pooled embryos representative of all vitrified groups were stained after 2 d of culture. Embryos were fixed in a solution of 3 methanol: 1 glacial acetic acid then stained with 1 % aceto-orcein stain. Fresh and vitrified embryos were utilized for cell counts. The number of blastomeres was determined by counting the number of nuclei and the nuclear stage was registered. Total number of 32 fresh embryos was used as a control.

2..5. Evaluation of apoptosis

At 48 h of culture after warming, embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) for 1 h at room temperature. After fixation, embryos were washed at least three times in PBS containing 0.3 % polyvinylpyrrolidone (PVP) and permeabilized in 0.5 % Triton X-100 on ice for 2 min. The embryos were then washed three times in PBS/PVP and incubated in TUNEL reaction cocktail (In-situ cell death detection system; Roche Diagnostic Corp., Indianapolis, IN, USA) at 37°C for 1 h in the dark. Positive and negative control samples were included in each analysis. Blastocysts exposed to DNAse 1 for 15 min at room temperature served as positive control. Blastocysts that were not exposed to the TdT enzyme served as negative control. Embryos were extensively washed in PBS and propidium iodide was used for counterstaining. Drops of mounting medium were covered with a cover slip, and the edges were sealed with clear nail polish and stored at -20°C in the dark for analysis, which was usually performed within 3 weeks. Positive reactivity was indicated by bright green/yellow fluorescence indicating apoptotic cells and the cells of embryos were determined by red fluorescence. Fresh embryos were used as controls.

2.6. Statistical analysis

Data were subjected to ANOVA using SPSS for Windows version 13.0, statistical software. Comparison of means was carried out by LSD**.** Differences were considered to be significant at $P<0.05$. Total cell numbers of fresh and vitrified embryos were analyzed by simple t-test.

3. Results

Results of blastocysts vitrification by ultra-rapid cooling (cryloop and cryotop) and rapid cooling procedures (straw in goblet and straw in Hasler device) are shown in table 1. Survival rates directly after warming were not significantly differ ($P<0.2$) between the four methods. There were significant differences ($P<0.05$) in survival rate after 24 h of culture among the methods. Cryloop group was poorer than other vitrification devices in percentage of viable embryos after 24 h. There was no statistical difference in hatched percentages between the different methods used in this study. Cryotop was best in the percentage of hatching but not significantly better than other cryodevices.

Table 1: Viability and hatched percentages of cattle blastocysts after vitrification and warming by different methods.

Vitrification methods	No. of vitrified-warmed blastocysts	No. of embryos which maintained viability (%)		Hatched after 48 h (%)
		After warming	After 24 hr	
Cryotop	54	46 (88.13)	37 (68.11) [a]	10.73
Cryoloop	35	26 (74.41)	18 (52.86) [b]	3.33
Straw in vapour	34	30 (90.00)	23 (67.29)	6.06
Straw in Hasler	32	28 (89.63)	21 (65.27) [a]	6.38
Total	155	130 (85.74)	99 (63.74)	6.94

Values with different superscripts within the same column differ significantly ($p<0.05$). No. = number.

Irrespective of vitrification methods, pooled embryos from the four methods were fixed and stained for counting the cell number. A significant ($P<0.001$) decrease in total cell number was observed in vitrified blastocysts than fresh embryos. In vitrified expanded and hatched blastocysts, the cell number was significantly increased and mitotic cells were significantly decreased compared to fresh embryos (Table, 2).

Table 2: Mean number of blastomeres and percentages of mitotic cells per embryo with 1-3 quality cattle blastocysts after vitrification.

Embryos	Blastocyst			Expanded and hatching blastocyst			Total embryos
	Embryos No.	Blastomeres No.	Mitotic cells %	Embryos No.	Blastomeres No.	Mitotic cells %	
Fresh	20	65.05*	10.42	12	114.75	7.39*	32
Vitrified	15	36.38	8.71	10	199.8*	2.58	25

*= significant ($P<0.001$)

The average number of apoptotic cells/embryo is shown in table 3. A significant increase in the number of apoptotic cells was recorded in all vitrification methods compared with fresh control embryo. Comparing the different methods of vitrification, cryotop and straws in goblet showed a significant decrease in apoptotic cells than the cryoloop and straws in the Hasler device.

Table 3: The occurrence of apoptosis and the average number of apoptotic cells per embryo after vitrification and warming by different methods.

Vitrification methods	Total number of embryos	Average No. of apoptotic cells/ embryo
Cryotop	10	13.50 [a]
Cryoloop	13	18.00 [a c]
Straw in vapour	13	14.30 [a]
Straw in Hasler	12	16.83 [a c]
Fresh	11	2.45 [b]

Values with different superscripts within the same column differ significantly ($p<0.001$). No.=number.

4. Discussion

Recent progress in the development of protocols for cryopreservation indicates that vitrification at the ultrarapid cooling rate is a promising approach for oocytes and embryos (Kuleshova and Lopata, 2002 and Attanasio et al. 2009). In the present study, after vitrification and warming, we tested the embryos from the four methods by culturing for further 24 h and 48 h to evaluate the survival and development. Embryo culture can be a useful method to assess viability and to confirm the quality of thawed embryos previously stored in liquid nitrogen (Contreras et al., 2008). The present study revealed that vitrification of bovine embryos by the four methods was similarly effective on viability after warming. Oberstein et al. (2001) reported no differences between the OPS, cryloop and conventional slow cooling for cryopreservation of equine embryos. Begin et al. (2003) stated that, the immediate survival rates of in vivo-

derived embryos vitrified by cryloop and solid-surface vitrification were not significantly different.

In our study, cryotop was best in the percentage of hatching but not significantly better than other cryodevices. Cryotop, straw in vapour and Hasler groups survive better after 24 h as compared to the cryoloop group. Cryotop and straw in vapour had significantly decreased apoptotic cells than cryoloop and straw in the Hasler device. In this respect, Hochi et al. (2004) reported that the ultra-rapid cooling with cryotop yielded higher post-thaw survival of rabbit zygotes than either GL-tip or cryoloop. Recently, cryotop vitrification was an efficient method for vitrification of human oocytes and embryos (Kuwayama, 2007), cattle and buffalo embryos (Laowtammathron et al., 2005) and bovine oocytes (Morato et al., 2008). Cryotop technology is a version of the minimal volume approaches which increases the cooling and warming rates (up to 40, 000°C /min) which may contribute to the improved survival and developmental rates (Kuwayama, 2007). Minimum volume vitrification methods as cryotop and cryloop may be helpful to avoid zona pellucida and embryo fracture damage. This type of injury occurs frequently when samples are cryopreserved in standard insemination straws and warmed rapidly afterwards. By using small samples and especially with vitrification in open systems, fracture damage rarely occurs, and it can be entirely eliminated with appropriate adjustment of warming parameters (Kuwayama, 2007). Also the Hasler vitrification device was modified to minimize the fracture occurring in straw with vapour.

The number of blastomeres represents the cleaving status of the embryo. The good quality embryo has many blastomeres (Baczkowski et al., 2004). In our work, decreases in total cell number were recorded in vitrified blastocysts than controls. Similarly, a decrease in total cell number was observed in both frozen and vitrified embryos (Kaidi et al., 2001). Cells probably degenerated quickly during the vitrification procedure. This could be explained by necrosis and apoptosis of cells during the cryopreservation procedure and / or following the procedure as demonstrated by Baguisi et al. (1999). This decrease, however, could also be due to a slowing in embryonic cell proliferation after cryopreservation (Takagi et al., 1996). The decrease in total cell number of embryos after vitrification leads to a disturbance in the hatching process and this explain the low rate of hatching after vitrification. In our results, the increase in the total cell number of hatched blastocyst in vitrified embryos than control may be due to differences in age of embryos, and this was probably due to difference in quality as the mitotic cells were significantly decreased in vitrified expanded and hatched blastocysts in this study. The large number of cells in vitrified expanded and hatching blastocyst indicated the efficiency of the techniques of vitrification in this study. It is thought that embryos with a large number of cells are more likely to implant and give rise to live offspring (Van Soom et al., 1997).

The amount of apoptosis in embryos is important parameter of embryo vitrification. In the present study, cells were evaluated by TUNEL assay, and TUNEL positive cells were found after 48 h in all embryos vitrified by the four methods. It was statistically significant compared to the control. These results agree with those by Marquez-Alvarado et al. (2004) who found TUNEL-positive cells in frozen embryos evaluated in different stages of development. Comparing the different methods of vitrification in our study, cryotop and straw in goblet showed significant decreases in apoptotic cells than cryoloop and straws in the Hasler device. This result confirmed the bad quality of embryos after 24 h post warming in cryloop compared by the other methods. In this respect, Contreras et al. (2008) found that frozen embryos classified as being of good quality had fewer TUNEL-positive cells than fair or poor quality embryos.

In conclusion, the four methods of vitrification had a relatively similar effect on viability directly after warming and hatching rate at 48 h. Cryotop, straws in vapour and Hasler device groups survived better after 24 h compared to the cryoloop group. Cryotop and straw in vapour had significant decreased in apoptotic cells than cryoloop and straws in the Hasler device.

Acknowledgments

The authors are very grateful to Zella Brink at the Embryo Transfer Laboratory at Colorado State University for her excellent technical assistance of the experiments.

Corresponding to:
K Gh M Mahmoud
Dept.Anim.Reprod.& A.I,
National Research Center
Dokki, Tahrir Street, 12622 Giza, Egypt
Tel.: 002 0101827716; Fax: 002 02 33370931
E.mail : karima_mahmoud@hotmail.com

References

[1] Attanasio L, Boccia L, Vajta G, Kuwayama M, Campanile G, Zicarelli L, Neglia G, Gasparrini B. Cryotop vitrification of buffalo (Bubalus Bubalis) in vitro matured oocytes: Effects of cryoprotectant concentrations and warming procedures. Reprod Domest Anim 2009;(in press).

[2] Baczkowski T, Kurzawa R, Glabowski W. Methods of embryo scoring in *in vitro* fertilization. Reproductive Biology 2004; 4: 5-22.

[3] Baguisi A, Lonergan P, Overstrom E W, Boland M P. Vitrification of bovine embryos: incidence of necrosis and apoptosis. Theriogenology 1999; 51: 162 (abstract).

[4] Begin I, Bhatia B, Baldassarre H, Dinnyes A, Keefer C L. Cryopreservation of goat oocytes and in vivo derived 2- to 4-cell embryos using the cryoloop (CLV) and solid-surface vitrification (SSV) methods. Theriogenology 2003; 59:1839-1850.

[5] Delval A, Ectors F J, Touati K, Beckers JF, Ectors, F. Vitrification of bovine embryos produced in vitro: survival, hatching and pregnancy rates. Theriogenology 1996; 45: 178 (Abstract).

[6] Campos-Chillon LF, Walker DJ, De La Torre-Sanchez , Seidel Jr GE. In vitro assessment of a direct transfer vitrification procedure for bovine embryos. Theriogenology 2006; 65: 1200–1214.

[7] Contreras DA, Galina CS, Avila JG, Aspron MP, Moreno-Mendozad, N. A system to evaluate the quality of frozen embryos through short-term culture. Anim Reprod Sci 2008; 106: 369–379.

[8] Fabian D, Koppel J, Maddox-Hyttel, P. Apoptotic processes during mammalian preimplantation development. Theriogenology 2005; 64: 221-231.

[9] Fahrudin M, Otoi T, Karja N, Mori M, Murakami M, Suzuki T. Analysis of DNA fragmentation in bovine somatic transfer embryos using TUNEL. Reproduction, 2002; 124: 813 – 819.

[10] Gomez E, Rodriguez A, Munoz M, Caamano J N, Hidalgo C O, Moran E, Facal N, Diez, C. Serum free embryo culture medium improves in vitro survival of bovine blastocysts to vitrification. Theriogenology 2008; 69: 1013-1021.

[11] Hochi S, Terao T, Kamei M, Kato M, Hirabayashi M, Hirao M. Successful vitrification of pronuclear-stage rabbit zygotes by minimum volume cooling procedure. Theriogenology 2004; 61: 267 – 275.

[12] Hufana- Duran D , Pedro P B , Venturina H V, Hufana R D, Salazar A L, Duran PG, Cruz L C. Post-warming hatching and birth of live calves following transfer of *in vitro*-derived vitrified water buffalo (Bubalus bubalis) embryos. Theriogenology 2004; 61: 1429 – 1439.

[13] Kaidi S, Bernard S, Lambert P, Massip A, Dessy F, Donnay I. Effect of conventional controlled-rate freezing and vitrification on morphology and metabolism of bovine blastocysts produced in vitro Biol Reprod 2001; 65: 1127–1134.

[14] Kasai M, Komi JH, Takakamo K, Isudera H, Sakurai T, Machida TA. Simple method for mouse embryo cryopreservation in a low toxicity vitrification solution, without appreciable loss of viability. J Reprod Fertil 1990; 89: 91–97.

[15] Kuleshova L L, Lopata A. Vitrification can be more favorable than slow cooling. Fertility and Sterility 2002; 78:449-454.

[16] Kuwayama M. Highly efficient vitrification for cryo- preservation of human oocytes and embryos: the Cryotop method. Theriogenology 2007; 67: 73–80.

[17] Kuwayama M, Kato O. All-round vitrification method for human oocytes and embryos. J Assist Reprod Genet 2000; 17: 477 (abstr.).

[18] Lane M , Schoolcraft W B, Gardner K D, Phil D. Vitrification of mouse and human blastocysts using a novel cryoloop container-less technique. Fertility and Sterility 1999a; 72: 1073-1078.

[19] Lane M, Bavister B D, Lyons E A, Forest K T. Containerless vitrification of mammalian oocytes and embryos. Nat. Biotechnology 1999b; 17: 1234-1236.

[20] Laowtammathron C, Lorthongpanich C, Ketudat-Cairns M, Hochi S, Parnpai R. Factors affecting cryosurvival of nuclear-transferred bovine and swamp buffalo blastocysts: effects of hatching stage, linoleic acid–albumin in IVC medium and Ficoll supplementation to vitrification solution. Theriogenology 2005; 64: 1185–1196.

[21] Lonergan p, Rizos D, Ward F, Boland M P. Factors influencing oocyte and embryo quality in cattle. Reprod Nutr Dev 2001; 41: 427-437.

[22] Marquez-Alvarado Y C, Galino CS, Castilla B, Leon H, Moreno-Mendoza N. Evidence of damage in cryopreserved embryos stored under tropical conditions using the TUNEL technique. Reprod Domest Anim 2004; 39: 141-145.

[23] Mazur P, Leibo SP, Seidel GE. Cryopreservation of the germplasm of animals used in biological and medical

research: importance, impact, status, and future directions. Biol Reprod 2008; 78: 2–12.

[24] Morato´ R, Izquierdo D, Paramio M T, Mogas T. Cryotops versus open-pulled straws (OPS) as carriers for the cryopreservation of bovine oocytes: Effects on spindle and chromosome configuration and embryo development. Cryobiology 2008; 57: 137–141.

[25] Mukaida T, Nakamura S, Tomiyama T, Wada S, Kasai M., Takahashi K. Successful birth after transfer of vitrified human blastocysts with use of a cryoloop containerless technique. Fertility and Sterility, 2001; 76: 618-620.

[26] Mukaida T, Takahashi K, Kasai M. Blastocyst cryopreservation: ultrarapid vitrification using cryoloop technique. Reprod Biomed Online 2003; 6:221-225.

[27] Niemann H. Cryopreservation of ova and embryos from livestock: current status and research needs. Theriogenology 1991; 35: 109 – 124.

[28] Oberstein N, O'Donovan MK, Bruemmer JE, Seidel GE Jr, Carnevale EM, Squires EL. Cryopreservation of equine embryos by open pulled straw, cryoloop, or conventional slow cooling methods. Theriogenology 2001; 55: 607–613.

[29] Olson SE, Seidel Jr GE. Reduced oxygen tension and EDTA improve bovine zygote development in a chemically defined medium. J Anim Sci 2000; 78:152–157.

[30] Parrish JJ, Susko-Parrish J, Winer MA, First NL. Capacitation of bovine sperm by heparin. Biol Reprod 1988; 38:1171–1180.

[31] Schiewe MC, Rall WF, Stuart LD, Wildt DE. Analysis of cryoprotectant, cooling rate and in situ dilution using conventional freezing or vitrification for cryopreserving sheep embryos. Theriogenology 1991; 36: 279–293.

[32] Takagi M, Sakonju I, Suzuki T. Effects of cryopreservation on DNA synthesis in the inner cell mass of in vitro matured/ in vitro fertilized bovine embryos frozen in various cryoprotectants. J Vet Med Sci 1996; 58:1237-1238.

[33] Takagi Y, Sakamoto M, Yamaguchi S, Ota K, Jindo M. Vitrification of mouse blastocysts by the cryoloop- straw method. Theriogenology 2002; 57: 484 [abstract].

[34] Vajta G, Nagy ZP. Are programmable freezers still needed in the embryo laboratory? Review on vitrification. Reprod Biomed 2006; 12: 779–796.

[35] Van Soom A, Ysebaert M, de Kurif A. Relationship between timing of development, morula morphology and cell allocation to the inner cell mass and trophectoderm in in vitro produced embryo. Mol Reprod Dev 1997; 47: 47-56.

[36] Wurth Y A, van der Zee-Kotting W I, Dieleman S J, Bevers MM, Kruip Th A M. Presence of mitotic cells: a parameter of embryo quality. Animal Reproduction Science 1994; 35: 173-182.

[37] Wurth Y A, van der Zee-Kotting W I, Kruip Th A M, Dieleman S J, Bevers M M. Relation between macroscopic qualification of bovine embryos and number of blastomeres. 11 international Congress on Animal Reproduction and Artificial Insemination, Dublin, June, 26th -30th, Ireland 1988; Pp. 352.

15/9/2009