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Effects of vitrification of partially denuded bovine immature oocytes*

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In spite of many trials on bovine oocyte cryopreservation, their efficiency remains unsatisfactory, regardless of method used. Although a births of about 15 calves obtained after transfer of embryos developed from frozen or vitrified oocytes were reported by now, a total efficiency of oocyte cryopreservation, in terms of blastocyst rate obtained, reaches approximately a half of those reported for fresh oocytes. The authors' former research on mature bovine oocyte vitrification showed a possibility of obtaining a very high oocyte survival rate and development of up to 29.6% of blastocysts. In the present paper, reported and discussed are results of similar vitrification procedure, but employed for germinal vesicle stage (GV) of bovine oocytes. Immature cumulus-oocyte complexes (COCs) obtained from ovarian follicles were vortexed for short time to strip off a portion of cumulus cells. Vitrification was performed using microdroplet method in VS14 solution, after 12-15 min pre-equilibration and 30 or 45 s equilibration periods. Warming was accomplished in warm dilution medium, without sucrose. After *in vitro* maturation and fertilization, presumptive zygotes were cultured *in vitro* up to day 10. Depending on pre-equilibration parameters 5.3- 8.4% blastocysts developed from vitrified oocytes, that is significantly less compared to blastocyst ratio obtained from control, fresh oocytes (40.9%, P≤0.01, Fisher test). Although the ratio of blastocysts obtained from vitrified GV oocytes presented in this paper does not differ from the best results reported elsewhere, the autors suggest a need of further extensive research on bovine immature oocyte vitrification to obtain replicable, satisfactory efficiency.

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Achievement a high efficiency of bovine oocyte cryopreservation remains for years of great interest of researchers and practitioners working on *in vitro* production of bovine embryos and somatic cloning. However, bovine oocytes turned out to be highly sensitive to both chilling and exposing to cryoprotective agents. Thus, in spite of several attempts, the results of slow cooling, traditional vitrification (in 0.25 mL straws) or even minimum sample size (MSS, or microvolumetric) vitrification usually remained disappointing [Hamano *et al.* 1992, Hochi *et al.* 1998, Le Gal and Massip 1999, Diez *et al.* 2005], for review see: Gajda and Smorąg [2009], Saragusty and Arav [2010], Prentice and Anzar [2011], Diez *et al*. [2012]. Our results published 12 years ago [Papis *et al*. 2000], showing high developmental capability of vitrified metaphase II bovine oocytes (29.6% blastocyst rate was obtained) did not change much a general pessimistic perception of this subject. Approximately only 15 calves born after bovine oocyte cryopreservation was reported by now [Hamano *et al*. 1992, Vajta *et al*. 1998, Papis *et al*. 2000]. Surprisingly, a vast majority of them was obtained from immature oocytes, *i.e*. oocytes cryopreserved in germinal vesicle (GV) stage [Suzuki *et al*. 1996, Kubota *et al*. 1998, Vieira *et al*. 2002, 2008]. Immature oocytes were usually treated as an object better suitable for cryopreservation as having less number of vulnerable structures, such as condensed chromosomes and with genetic material protected by nuclear envelope [Luciano *et al*. 2009, Prentice and Anzar 2011]. However, general efficiency of their cryopreservation is rather low, regardless of method used (Tab. 1 and 2). The main possible reason for this is, that immature oocyte should be considered as a one functional unit with cumulus cells communicating each other *via* cellular projections penetrating across a *zona pellucida*. Undisturbed oocyte-cumulus communication and co-operation seem essential for adequate maturation process [Gilchrist 2011], so an optimum cryopreservation method should provide protection for oocyte, cumulus cells and their intercommunication system. Although immature oocytes vitrified without cumulus were matured and fertilized *in vitro* with acceptable efficiency [Modina *et al*. 2004, Luciano *et al*. 2009, Zhou *et al*. 2010], their further development as embryos was compromised [Modina *et al*. 2004]. Cumulus cells surrounding oocytes constitute multilayer tight barrier resulting in a kind of undesired "protection" of oocytes prepared for vitrification. Such barrier may delay saturation of oocytes by a proper amount of cryoprotective agents required for efficient vitrification [Papis 1996].

We proved already of high efficiency of the "in droplet" vitrification method for mature cumulus-free oocyte vitrification [Papis *et al*. 2000, Stachowiak *et al*. 2009]. In this method oocytes apparently took advantage from pre-equilibration step in diluted (3%) ethylene glycol solution, prior to final short exposure to the vitrification medium. The aim of experiments reported here was an evaluation of the same method used for vitrification of GV bovine oocytes partially denuded from cumulus cells.

Material and methods

Source of oocytes

Ovaries obtained from the local slaughterhouse were transported to the laboratory in saline at 30°C, within 2 to 3 h. Cumulus-oocyte complexes (COCs) were aspirated with follicular fluid through 18- gauge needle from follicles of 2 to 6 mm in diameter. Evenly granulated oocytes surrounded with multilayer of compacted cumulus cells were selected under stereomicroscope for experiments. Before vitrification COCs were partially denuded from cumulus cells (Fig. 1) by means of short vortexing in 0.025% hyaluronidase solution (SIGMA, St. Louis, MO) and kept in a portion of fresh medium until use.

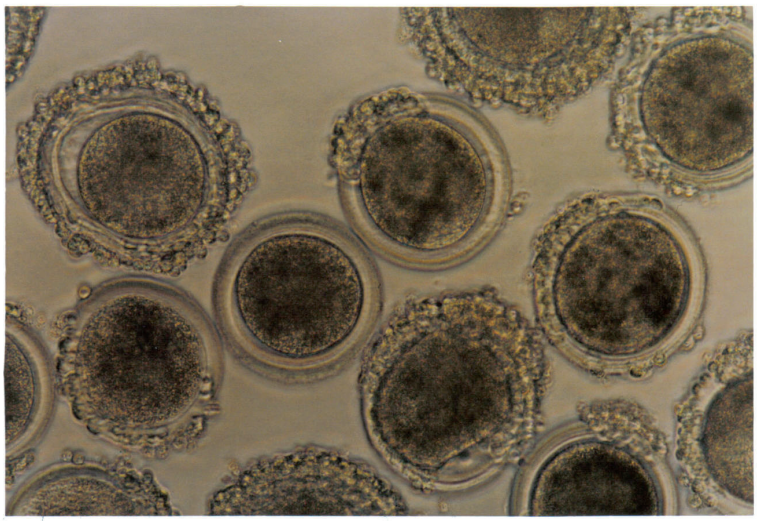

Fig. 1. Immature bovine oocytes vitrified after partial denuding from cumulus cells, directly after warming.

Vitrification

Vitrification was performed according to the droplet vitrification method described earlier [Papis *et al*. 1999, Papis *et al*. 2000] with minor modifications. Hepes-buffered TCM 199 medium (Earle's salts, GIBCO BRL, Grand Island, NY) supplemented with 20% FCS (TCM/20%FCS) was used as a basic solution throughout the experiments. Vitrification solution VS14 containing 5.5 M (31% v/v) ethylene glycol (SIGMA) and 1.0 M sucrose (SIGMA), [Ali and Shelton 1993] was employed both for a final equilibration and vitrification. Majority of procedures including pre-equilibration, equilibration, and warming/diluting were performed in 35-37°C.

Oocytes randomly allocated to treatments were subjected in groups of 12-18 to pre-equilibration in diluted (2 or 3% (v/v) ethylene glycol (EG) solution for 12-15

min. Subsequently, portions of 5-8 oocytes were transferred within 25-40 s through three changes of fresh VS14 vitrification solution for equilibration. Finally, oocytes were dropped to the styrofoam box filled with liquid nitrogen, directly from a Pasteur pipette kept about 15 cm above the liquid level. The total exposure time of oocytes to VS14 solution was 30 or 45 s depending on the experimental group. Vitrified droplets containing about 6μL (±2μL) of vitrification solution were collected to 0.75 mL cryotubes using fine precooled forceps and kept in liquid nitrogen for 1-2 hours. Warming was performed by means of direct immersing of each droplet in 35 mm Petri dish containing 2 mL of warm TCM/20%FCS medium (without sucrose) and gentle stirring. Then oocytes were collected and transferred to 2 mL of fresh TCM medium. Ten to 15 min after warming, the oocytes from each treatment group were rinsed in two changes of maturation medium and subjected to maturation procedure. Controls, not treated oocytes, were kept in fresh TCM medium under culture conditions until maturation.

Maturation, fertilization and *in vitro* culture

In vitro maturation and fertilization of oocytes was based on the method presented by Papis *et al*. [2000] with some modifications. Procedure was performed in TCM 199 Hepes buffered medium (GIBCO) supplemented with 1μg/mL oestradiol (SIGMA), 0.002 AU/mL FSH (Antrin, DENKA Pharmaceutical Industry, Kawasaki, Japan) and 5% FCS (CELL CULTURE TECHNOLOGIES, Canada). Oocytes were washed 3 times in fresh maturation medium and placed in 50 μL droplets covered with mineral oil (SQUIBB and SONS Inc., Princeton, NJ) for 23-25 h incubation at 38.5°C under 5% CO2 in air. Insemination of oocytes was performed using frozen sperm of one bull prepared in BO medium [Brackett and Oliphant 1975] supplemented with caffeine (SIGMA) and suspended in a concentration of about 5 x 10^6 spermatozoa/mL in 50 μL droplets of BO medium containing additionally BSA (SIGMA) and heparin (KODAMA, Tokyo, Japan). Modified CR1 aa medium [Rosenkrans and First 1991] containing BSA and linoleic acid was used for *in vitro* culture of zygotes. On Day 3, CR1 aa medium was supplemented with FCS. From Day 5 embryos were cultured in CR1 aa medium with FCS and glucose on a granulose cells monolayer. Culture was performed at 38.5^{O}C in 5% CO_2, 5% O_2 and high humidity. Cleavage, blastocyst and hatched blastocyst rates were scored.

Statistical

Means were computed of a number of oocytes vitrified/warmed. Significance of differences between proportions of embryos obtained from each treatment group was analysed using Fisher's exact test (InStat 3.01, GraphPad Software, Inc., San Diego USA). Values were considered statistically significant if $P \leq 0.05$.

Results and discussion

A total of 275 cumulus-oocyte complexes (COCs) were examined. Out of 83 COCs vitrified after equilibration in 2% EG and 45 s in VS14, 54.2, 8.4 and 7.2% cleaved and developed to blastocyst and hatched blastocyst rates, respectively (Tab. 3). Thirty second treatment in VS14 tended to be slightly less efficient, resulting in 47.9, 5.3, 3.2 and 57.1, 7.1 and 3.1% of cleavage, blastocyst and hatched blastocyst rates after equilibration in 2 and 3% EG solution, respectively (Fig. 2). On the other hand, 82.6, 40.9, and 23.5% of additional control, non-vitrified oocytes (n=115) cleaved and developed to blastocyst and hatched blastocyst rates, respectively ($P \leq 0.01$).

Results presented in this paper remain within the range of the best cryopreservation efficiency reported for years for immature bovine oocytes subjected to freezing [Suzuki *et al*. 1996, Kubota *et al*. 1998] or vitrification [Vieira *et al*. 2002, Modina *et al*. 2004, Abe *et al*. 2005, Zhou *et al*. 2010] – Tables 1 and 2. It suggests, that the procedure proposed here is moderately efficient, but still not fully optimal for oocytes cryopreserved in around germinal vesicle maturation stage. On the contrary, similar method, *i.e*. vitrification in droplets [Papis *et al*. 2000] or using cryo-loop [Checura and Seidel 2007] preceded by up to 15 min pre-equilibrations in diluted ethylene glycol solution was very efficient in cryopreservation of *in vitro* matured bovine oocytes [Papis *et al*. 2000, Checura and Seidel, 2007] and for *in vitro* produced Day 3 bovine embryos [Papis *et al*. 1999]. Many factors affecting the survival, fertilizability and developmental capacity of cryopreserved bovine oocytes were already described and discussed [Arav *et al*. 1996, Martino *et al*. 1996, Agca *et al.* 1998, Hochi *et al*. 1998,

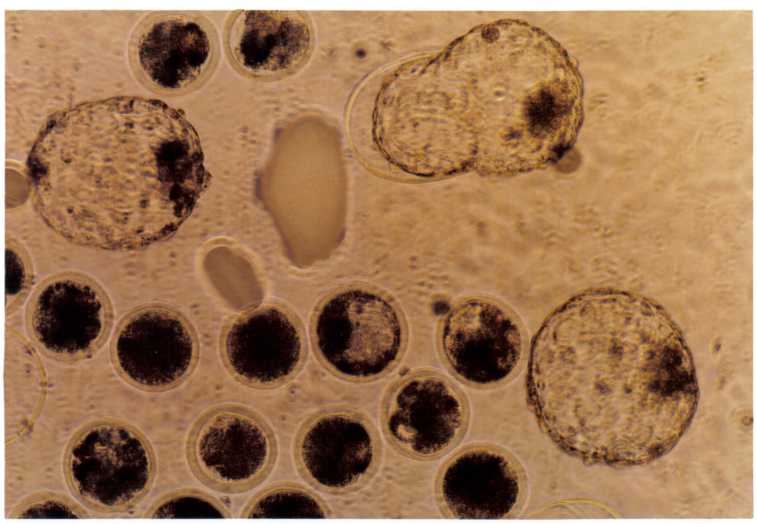

Fig. 2. Bovine day 9 blastocysts (2 hatched, 1 hatching) developed *in vitro* from vitrified immature oocytes.

Table 1. Early or traditional attempts at cryopreservation of bovine immature (germinal vesicle stage) oocytes

Method	Cryoprotectant	Results	Reference
Freezing	propanediol, trehalose	6% matured to M II	Heyman *et al*. [1986]
Vitrification	DMSO, acetamide, propanediol	1.1% cleavage rate	Fuku *et al*. [1992]
Freezing	propanediol	2% 2 cell embryos	Fuku *et al*. [1992]
Freezing	propanediol	13% cleavage rate	Schellander *et al*. [1994]
Freezing	ethylene glycol, sucrose	1% blastocyst rate, pregnant recipient	Otoi *et al*. [1995]
Freezing	ethylene glycol, trehalose, PVP	9.4% blastocyst rate, 3 calves	Suzuki *et al*. [1996]
Freezing	ethylene glycol, propanediol, sucrose	7% blastocyst rate, 2 calves	Kubota *et al*. [1998]
Vitrification	ethylene glycol, sucrose	34.1% matured to M II	Cetin & Bastan, [2006]
Freezing	ethylene glycol, sucrose	36.5% matured to M II	Luciano *et al*. [2009]

Table 2. Minimum Sample Size (MSS) methods of bovine immature oocyte vitrification

Method/holder	Cryoprotectant	Results	Author/Year
SSV, microdroplets	ethylene glycol, glycerol, galactose	3.3% blastocyst rate	LeGal and Massip [1999]
OPS	ethylene glycol, ficoll, sucrose	60% matured to MII	Hurtt *et al*. [2000]
Nylon mesh	ethylene glycol, ficoll, sucrose	3.4% 8-cell embryos	Matsumoto *et al*. [2001]
OPS	DMSO, ethylene glycol, sucrose	38.8 % matured to M II	Luna *et al*. [2001]
OPS	DMSO, ethylene glycol, sucrose	6.1% blastocyst rate, 3 calves	Vieira *et al*. [2002]
OPS	DMSO, ethylene glycol, sucrose	4.3% blastocyst rate	Modina *et al*. [2004]
OPS	DMSO, ethylene glycol, sucrose	2.8% embryos in morula stage	Diez *et al*. [2005]
Nylon mesh	ethylene glycol, ficoll, sucrose	8% blastocyst rate	Abe *et al*. [2005]
OPS	DMSO, ethylene glycol, trehalose	29.2% matured to M II	Yamada *et al.* [2007]
Microdroplets	DMSO, ethylene glycol, sucrose	2.3% blastocyst rate	Kim *et al*. [2007]
Glass micropipettes	DMSO, ethylene glycol, sucrose	5,9% blastocyst rate 4 calves born	Vieira *et al*. [2008]
OPS	DMSO, ethylene glycol, sucrose	76,2% M II (not different from control)	Luciano *et al*. [2009]
CryoTop	DMSO, ethylene glycol, sucrose	51.6% matured to M II	Hadi *et al*. [2010]
CryoTop	DMSO, ethylene glycol, sucrose	11.3% blastocyst rate	Zhou *et al*. [2010]
OPS	DMSO, ethylene glycol, trehalose	32,8% matured to M II	Yamada *et al.* [2011]
Microdroplets	ethylene glycol, sucrose	8.4% blastocyst rate	Papis *et al*. this study

SSV – solid surface vitrification; OPS – open pulled straw.

Table 3. Number and percentage (in brackets) of embryos developed from oocytes vitrified after pre-equilibration in different concentrations of ethylene glycol (EG) and exposure to VS14 vitrification solution for 30 or 45 s (means for 3 or 4 replicates).

Concentration of EG in pre-equilibration solution	Time of exposure to VS14 (s)	Number of treated oocytes	Cleaved	Developed to blastocyst stage	Developed to hatched blastocyst stage
2%	30	94	45 (47.9)[a]	5 (5.3)[a]	3 (3.2)[a]
	45	83	45 (54.2)[a]	7 (8.4)[a]	6 (7.2)[a]
3%	30	98	56 (57.1)[a]	10 (7.1)[a]	3 (3.1)[a]
Control (no vitrification)	-	115	95 (82.6)[b]	47 (40.9)[b]	27 (23.5)[b]

[ab]Within columns values bearing different superscript letters are significantly different at $P<0.01$.

Vajta *et al*. 1998, Modina *et al*. 2004, Stachowiak *et al*. 2009, Zhou *et al*. 2010, Prentice and Anzar 2011, Diez *et al*. 2012], including problems deriving from a need of deeper understanding of the crucial role of cumulus cell layers surrounding oocytes. Cumulus cells are closely related to oocytes, forming in fact one functional unit reflecting each other on auto- or paracrine manner [Gilchrist 2011]. Cumulus-oocyte interrelationship during oocyte maturation process seems crucial for achievement of optimum oocyte maturation and developmental capacity.

From earlier, preliminary experiments [Papis *et al*. 1995, Papis 1996], we were aware of key meaning of exposure time during which oocytes or COCs are subjected to highly concentrated vitrification solution(s). For mature oocytes denuded from cumulus cells, tendency was clear: 30 s exposition to vitrification solution was less detrimental to oocytes than 45 s [Papis *et al*. 2000]. On the other hand, denuded MII oocytes survived vitrification (in straws) better (44% survival rate regardless of 30 or 45 s VS14 exposition) than MII oocytes surrounded with expanded cumulus cells (3% and 18% survival rate after 30 and 45 s exposition, respectively) – Papis [1996]. We concluded from those results a possible need of longer COCs exposition or cumulus cells removal before exposition to cryoprotective agents. Consequently, considering additionally various thickness of cumulus cells layers surrounding oocytes, possibly affecting permeating rate of cryoprotective agents into (and out of) oocyte we were faced with multifactorial problem, rather difficult to solve. To deal in our experiment with, we vitrified GV oocytes partially denuded from cumulus cells (Fig. 1) in order to expose at least a part of oocyte surface to direct action of solutions. On the other hand, a portion of cumulus cells was left attached to the *zona pellucida* to facilitate adequate post-warming maturation and/or fertilization processes. Similar approach was presented recently by Zhou *et al*. [2010], who showed, that CryoTop vitrification of GV oocytes preceded by up to 1 min exposition to EG + DMSO vitrification solution may be quite efficient for cumulus enclosed GV oocytes, enabling development of 11% of blastocysts. On the other hand, development of partially denuded oocytes was inferior (4%) in their experiments. The reason of this effect remains unclear. However, from our earlier attempts [Papis *et al*. 1995] we conclude, that longer (1 min)

exposure of cumulus enclosed oocytes to vitrification solution(s) may be detrimental and this effect may become more prominent in case of denuded, or partially denuded oocytes.

As "partial denudation" results in variable effects, *i.e*. different denudation levels in terms of number of cumulus cells still attached to oocytes (Fig. 1), due probably to some intrinsic aspects of cumulus cell layers (tightness, *etc*) surrounding oocytes, it seems useful to consider a separate treatment of "more denuded" or "less denuded" oocytes prior to vitrification. It seems quite obvious, that conditions of equilibration should be precisely dedicated to particular class of partially denuded oocytes to be vitrified. On the other hand, precise analysis of pre- and equilibration conditions of intact COCs could help to select mostly appropriate timing and/or temperature conditions of these pre-vitrification steps. Alternatively, a research aimed at increasing of fully denuded bovine oocyte maturation efficiency would result in summarized improvement of vitrification effects. Taken together, additional extensive research is needed to achieve a final solving of problems observed in vitrification of bovine GV oocytes.

REFERENCES

1. ABE Y., HARA K., MATSUMOTO H., KOBAYASHI J., SASADA H., EKWALL H., RODRIGUEZ-MARTINEZ H., SATO E., 2005 – Feasibility of a nylon-mesh holder for vitrification of bovine germinal vesicle oocytes in subsequent production of viable blastocysts. ***Biology of Reproduction*** 72**,** 1416-1420.
2. AGCA, Y., LIU, J., PETER, A.T., CRITSER, E.S., CRITSER, J.K., 1998 – Effect of developmental stage on bovine oocyte plasma membrane water and cryoprotectant permeability characteristics. ***Molecular Reproduction and Development*** 49, 408-415.
3. ALI J., SHELTON JN., 1993 – Vitrification of preimplantation stages of mouse embryos. ***Journal of Reproduction and Fertility*** 98, 459-465.
4. ARAV A., ZERON Y., LESLIE S.B., BEHBOODI E., ANDERSON G.B., CROWE, J.H., 1996 – Phase transition temperature and chilling sensitivity of bovine oocytes. ***Cryobiology*** 33, 589-599.
5. ARAV A., ZERON Y., 1997 – Vitrification of bovine oocytes using modified minimum drop size technique (MDS) is affected by the composition and the concentration of the vitrification solution and by the cooling conditions. ***Theriogenology*** 47, 341 (abstract).
6. BRACKETT B.G., OLIPHANT G., 1975 – Capacitation of rabbit spermatozoa in vitro. ***Biology of Reproduction*** 12, 260-274.
7. CETIN Y., BASTAN A., 2006 – Cryopreservation of immature bovine oocytes by vitrification in straws. ***Animal Reproduction Science*** 92, 29-36.
8. CHECURA C.M., SEIDEL G.E.Jr,. 2007– Effect of macromolecules in solutions for vitrification of mature bovine oocytes. ***Theriogenology*** 67, 919-930.
9. DIEZ C., DUQUE P., GÓMEZ E., HIDALGO C.O., TAMARGO C., RODRÍGUEZ A., FERNÁNDEZ L., DE LA VARGA S., FERNÁNDEZ A., FACAL N., CARBAJO M,. 2005 – Bovine oocyte vitrification before or after meiotic arrest: effects on ultrastructure and developmental ability. ***Theriogenology*** 64, 317-333.
10. DIEZ C., MUNOZ M., CAAMANO JN., GOMEZ E., 2012 – Cryopreservation of bovine oocytes: current status and perspectives. ***Reproduction in Domestic Animals*** 47 (Suppl. 3), 76-83.
11. FUKU E, KOJIMA T, SHIOYA Y, MARCUS G.J, DOWNEY B.R,. 1992 – In vitro fertilization and development of frozen-thawed bovine oocytes. ***Cryobiology*** 29, 485-492.

12. GAJDA B., SMORĄG Z., 2009 – Oocyte and embryo cryopreservation – state of art and recent developments in domestic animals. ***Journal of Animal and Feed Sciences*** 18, 371-387.
13. GILCHRIST, R.B., 2011 – Recent insights into oocyte-follicle cell interactions provide opportunities for the development of new approaches to in vitro maturation. ***Reproduction Fertility and Development*** 23, 23-31,
14. HADI H., WAHID H., ROSNINA Y., DALIRI M., DASHTIZAD H., KARAMISHABANKAREH H., FAIZAH A., ISWADI M.I., ABAS MAZNI O., 2010 – Selection of immature bovine oocytes using brilliant cresyl blue enhances nuclear maturity after vitrification. ***Journal of Animal and Veterinary Advances*** 9, 2710-2713.
15. HAMANO S., KOIKEDA A., KUWAYAMA M., NAGAI T., 1992 – Full-term development of in vitro-matured, vitrified and fertilized bovine oocytes. ***Theriogenology*** 38, 1085-1090.
16. HEYMAN Y, SMORĄG Z, KATSKA L, VINCENT L., 1986 – Influence of carbohydrates, cooling and rapid freezing on viability of bovine non-matured oocytes or 1-cell fertilized eggs. ***Cryo-letters*** 7, 170-183.
17. HOCHI S., ITO K., HIRABAYASHI M., UEDA M., KIMURA K., HANADA A., 1998 – Effect of nuclear stages during IVM on the survival of vitrified-warmed bovine oocytes. ***Theriogenology*** 49, 787-796.
18. HURTT A.E., LANDIM-ALVARENGA F., SEIDEL G.E., Jr,. SQUIRES E.L., 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.
19. IM K.S., KANG J.K., KIM H.S,. 1997 – Effects of cumulus cells, different cryoprotectants, various maturation stages and preincubation before insemination on developmental capacity of frozen-thawed bovine oocytes. ***Theriogenology*** 47, 881-891.
20. KIM D-H., PARK H-S., KIM S-W., HWANG I-S., YANG B-C., IM G-S., CHUNG H-J., SEONG H-W., MOON S-J., YANG B-S., 2007 – Vitrification of immature bovine oocytes by the microdrop method, ***Journal of Reproduction and Development*** 53, 843-851.
21. KUBOTA C., YANG X., DINNYES A., TODOROKI J., YAMAKUCHI H., MIZOSHITA K., INOHAE S., TABARA N., 1998 – In vitro and in vivo survival of frozen-thawed bovine oocytes after IVF, nuclear transfer and partenogenetic activation. ***Molecular Reproduction and Development*** 51, 281-286.
22. LE GAL F., MASSIP A., 1999 – Cryopreservation of cattle oocytes: effects of meiotic stage, cycloheximide treatment and vitrification procedure. ***Cryobiology*** 38, 290-300.
23. LUCIANO A.M, FRANCIOSI F., LODDE V., PERAZZOLI F., SLEZAKOVA M., MODINA S., 2009 – Cryopreservation of immature bovine oocytes to reconstruct artificial gametes by germinal vesicle transplantation. ***Reproduction in Domestic Animals*** 44, 480-488.
24. LUNA H.S., FERRARI I., RUMPF R., 2001 – Influence of stage of maturation of bovine oocytes at time of vitrification on the incidence of diploid metaphase II at completion of maturation. ***Animal Reproduction Science*** 68, 23-28.
25. MODINA S., BERETTA M., LODDE V., LAURIA A., LUCIANO A.M., 2004 – Cytoplasmic changes and developmental competence of bovine oocytes, cryopreserved without cumulus cells. ***European Journal of Histochemistry*** 48, 337-346.
26. MARTINO A., SONGSASEN N., LEIBO S.P., 1996 – Development into blastocysts of bovine oocytes cryopreserved by ultra-rapid cooling. ***Biology of Reproduction*** 54, 1059-1069.
27. MATSUMOTO H., JIANG J. Y., TANAKA T., SASADA H., SATO E., 2001 – Vitrification of large quantities of immature bovine oocytes using nylon mesh. ***Cryobiology*** 42, 139-144.
28. OTOI T., YAMAMOTO K., KOYAMA N., SUZUKI T., 1995 – In vitro fertilization and development of immature and mature bovine oocytes cryopreserved by ethylene glycol with sucrose. ***Cryobiology*** 32, 455-460.

29. PAPIS K., 1996 – The effect of cumulus cells on survivability of vitrified bovine in vitro matured oocytes. Proceedings of the 13th International Congress of Animal Reproduction, Sydney, Australia. vol. 3, P15-17.
30. PAPIS K., AVERY P., HOLM P., CALLESEN H., GREVE T., 1995 – The effect of vitrification solution, equilibration time and direct dilution method on survivability of equilibrated or vitrified bovine in vitro matured oocytes. ***Theriogenology*** 43, 293 (abstract).
31. PAPIS, K., SHIMIZU, M., IZAIKE, Y., 1999 – A highly efficient, modified vitrification method, for Day 3 in vitro produced bovine embryos ***Cryo-Letters*** 20, 203-206.
32. PAPIS, K., SHIMIZU, M., IZAIKE, Y., 2000 – Factors affecting the survivability of bovine oocytes vitrified in droplets. ***Theriogenology*** 54, 651-658.
33. PRENTICE, J.R., ANZAR, M., 2011 – Cryopreservation of Mammalian Oocyte for Conservation of Animal Genetics. ***Veterinary Medicine International*** Article ID 146405, 11 pages, 2011. doi:10.4061/2011/146405.
34. ROSENKRANS F., FIRST N.L., 1991 – Culture of bovine zygotes to the blastocyst stage: effects of amino acids and vitamins. ***Theriogenology*** 35, 266 (abstract).
35. SARAGUSTY J., ARAV A., 2011 – Current progress in oocyte and embryo cryopreservation by slow freezing and vitrification. ***Reproduction*** 141, 1-19.
36. SCHELLANDER K., PELI J., SCHMOLL F., BREM G., 1994 – Effects of different cryoprotectants and carbohydrates on freezing of mature and immature bovine oocytes. ***Theriogenology*** 42, 909-915.
37. STACHOWIAK E.M., PAPIS K., KRUSZEWSKI M., IWANENKO T., BARTŁOMIEJCZYK T., MODLINSKI J.A., 2009 – Comparison of the level(s) of DNA damage using comet assay in bovine oocytes subjected to selected vitrification methods ***Reproduction in Domestic Animals*** 44, 653-658.
38. SUZUKI T., BOEDIONO A., TAKAGI M., SAHA S., SUMANTRI C., 1996 – Fertilization and development of frozen-thawed germinal vesicle bovine oocytes by one-step dilution method in vitro. ***Cryobiology*** 33, 515-524.
39. VAJTA G., HOLM P., KUWAYAMA M., BOOTH P.J., JACOBSEN H., GREVE T., CALLESEN H., 1998 – Open Pulled Straw (OPS) vitrification: A new way to reduce cryoinjuries of bovine ova and embryos. ***Molecular Reproduction and Development*** 51, 53-58.
40. VIEIRA A.D., FORELL F., FELTRIN C., RODRIGUES J.L., 2008 – Calves born after direct transfer of vitrified bovine in vitro-produced blastocysts derived from vitrified immature oocytes. ***Reproduction in Domestic Animals*** 43, 314-318.
41. VIEIRA A.D., MEZZALIRA A., BARBIERI D.P., LEHMKUHL R.C., RUBIN M.I.B., VAJTA G., 2002 – Calves born after open pulled straw vitrification of immature bovine oocytes. ***Cryobiology*** 45, 91-94.
42. YAMADA C., CAETANO V.H.A., SIMOES R., NICACIO A.C., FEITOSA W.B., ASSUMPCAO M.E.O.A., VISINTIN J A., 2007– Immature bovine oocyte cryopreservation: Comparison of different associations with ethylene glycol, glycerol and dimethylsulfoxide ***Animal Reproduction Science*** 99, 384-388.
43. YAMADA C., FEITOSA W.B., SIMOES R., NICACIO A.C., MENDES C.M., ASSUMPCAO M.E.O.A., VISINTIN J.A., 2011 – Vitrification with glutamine improves maturation rate of vitrified/warmed immature bovine oocytes. ***Reproduction in Domestic Animals*** 46, 173-176.
44. ZHOU X.L., AL NAIB A., SUN D.W., LONERGAN P., 2010 – Bovine oocyte vitrification using the Cryotop method: Effect of cumulus cells and vitrification protocol on survival and subsequent development. ***Cryobiology***, 61, 66-72.