ERLY LUISANA CARRASCAL TRIANA

EFFECTS OF ANTIOXIDANTS ASCORBIC ACID AND DITHIOTHREITOL, OR AN INHIBITOR OF CASPASE-3 DURING CRYOPRESERVATION OF IN VITRO PRODUCED BOVINE EMBRYOS

Thesis submitted to the Animal Science Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of Doctor Scientiae.

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APPROVED: September 29th, 2016

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Ciro Alexandre Alves Torres
(Adviser)

"Yesterday is but a dream, tomorrow is only a vision.

But today well lived makes every yesterday a dream of happiness,

and every tomorrow a vision of hope."

Kalidasa

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BIOGRAPHY

Erly Luisana Carrascal Triana, daughter of Pedro Carrascal Carrascal and Amparo Triana Dominguez, was born and raised in Montelíbano, Cordoba, Colombia. In 2005, began her studies at the University of Cordoba in Monteria, Cordoba, Colombia. She did a training in the Animal Reproduction Laboratory at the Universidade Federal de Viçosa (UFV), Minas Gerais, Brasil from August 2009 to February, 2010 under supervision of Dr. José Domingos Guimaraes and she graduated in Veterinary Medicine and Animal Science in December 2010. After this period, she returned to the UFV - Brasil to work under Dr. Ciro Alexandre Alves Torres on her Master of Science with Manipulation of ovarian preantral follicles and she graduated in July 2012. Erly was trainee in the Laboratory of Oocytes and Preantral Ovarian Follicles Manipulation (in Portuguese, LAMOFOPA) under the supervision of Dr. José Ricardo Figueiredo, and after that she did a training in Biotechnology and Animal Reproduction (in Portuguese BIOTRAN) under the supervision of Dr Carlos Antonio de Carvalho Fernandes. In 2013 she moved to the UFV to start her doctorate studies in the Animal Science Graduate Program under the supervision of Dr. Ciro A.A. Torres. During three years at UFV, she was supported by a Graduate Fellowship and work with in vitro embryo production (in vitro maturation, fertilization, embryo development and embryo cryopreservation). From August to December 2014, Erly took a trainee stage at Florida University, Gainesville, under Dr. Jeremy Block adviser and during this time she did some experiments focused on improving the cryotolerance in vitro produced bovine embryos.

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LIST OF ABBREVIATIONS

Abbreviation	Term (Alias)
AA	Ascorbic Acid
AA2G	Ascorbic acid 2-O-α-glucoside
ATP	Adenosine triphosphate
BARC	Betsville Agriculture Research Center medium
COCs	Cumulus Oocyte Complexes
DAPI	4′,6-diamidino-2-phenylindole
DIC	Differential interference contrast
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
EG	Ethylene Glycol
FITC	Fluorescein isothiocyanate
FSH	Follicle-stimulating hormone
GLM	General Linear Model
GLUTs	Glucose transporters
GPX1	Glutathione peroxidase 1
GSH	Glutathione
GSSG	Glutathione disulfide
HSPA1A	Heat shock 70 kDa protein 1
Hepes	Hydroxyethylpiperazine Ethanesulfonate
IVC	in vitro Culture
IVM	in vitro Maturation
IVP	in vitro production
TIMP-2	Metalloproteinase inhibitor 2 precursor
MMP-9	Matrix metalloproteinase-9
PBS	Phosphate-buffered saline
PVP	Polyvinylpyrrolidone
ROS	Reactive Oxygen Species
REDOX	Reduction–oxidation reaction
SAS	Statistical Analysis System
SEM	Standard Error of Mean

SOD1	Superoxide dismutase 1
SOF-BE1	Synthetic Oviductal Fluid-Bovine Embryo 1
SUC	Sucrose
SVCTs	Sodium ascorbate cotransporter
TALP	Tyrodes Albumin Lactate Pyruvate
TUNEL	Terminal Deoxynucleotidyl Transferase Deoxyuridine Triphosphate Nick and Labeling
UDP	Uridine diphosphate
z-DEVD-fmk	N-Benzyloxycarbonyl-Asp-Glu-Val-Asp-Fluoromethyl Ketone

ABSTRACT

CARRASCAL-TRIANA, Erly Luisana, D.Sc., Universidade Federal de Viçosa, September, 2016. **Effects of antioxidants ascorbic acid and dithiothreitol, or an inhibitor of caspase-3 during cryopreservation of in vitro produced bovine embryos.** Adviser: Ciro Alexandre Alves Torres.

Experiments described in this study were performed with the overall objective to evaluate whether the addition of antioxidants ascorbic acid (AA) and dithiothreitol (DTT), or inhibitor of caspase-3 (z-DEVD-fmk) during cryopreservation could improve the cryotolerance of in vitro produced bovine embryos. Five experiments were performed and represented as follows: experiment 1, 2 and 3 in Chapter 2 and experiment 4 and 5 in Chapter 3. For all experiments, cumulus oocyte complexes were obtained from ovaries of slaughterhouse cows. Oocytes were in vitro matured, fertilized and cultured to day 7. Blastocysts and expanded blastocysts were randomly assigned to be subjected to controlled-rate freezing following equilibration for 10 min in freezing medium (Hepes-TALP plus 1.5 M ethylene glycol and 0.1 M Sucrose) with treatments as described below. For experiment 1, the embryos were equilibrated in freezing medium containing 0.0; 0.1; 0.3 or 0.5 mM of AA. The embryos into straws were then placed into programmable freezing machine at -6.0 °C to -32 °C prior to being plunged into liquid nitrogen (-196 °C). Then, embryos were thawed and cultured for 72 h in SOF-BE1 supplemented with 10% (v/v) fetal bovine serum at 38.5 ºC in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2. Embryos treated with 0.1 mM AA showed higher re-expansion at 24, 48 and 72 h and hatching rates at 72 h compared to control embryos ($P<0.05$), thus concentration was considered as the optimal for the sequential experiments. For experiment 2 and 3, embryos were cryopreserved in freezing medium containing or not 0.1 mM AA, then were thawed and cultured for 24 h. Intracellular reactive oxygen species levels were reduced ($P<0.001$) by AA treatment (30.3 ± 2.4) compared with control (49.3 ± 1.9). There was no effect of the total cells number of blastocyst ($P>0.05$). However, 0.1 mM AA reduced ($P<0.001$) the percentage of apoptotic cells and DNA fragmentation compared with the control group. Experiments 4 and 5 were conducted to assess the effects of DTT or z-DEVD-fmk during cryopreservation. Blastocyst and expanded blastocysts embryos were equilibrated in freezing medium containing DTT (0, 50, 100 and 200 µM) or z-DEVD-fmk (0, 50, 100 and 200 µM).

The embryos into straws were then placed into programmable freezing machine at -6.0 °C to -32 °C prior to being plunged into liquid nitrogen (-196 °C). Embryos were thawed and then cultured for 72 h. Re-expansion and hatching rates were recorded at 24, 48 and 72 h. There was no effect ($P>0.05$) of treatment with DTT or z-DEVD-fmk on re-expansion or hatching rates at 24, 48 or 72 h post-thaw. This is the first report that used AA and DTT antioxidants or z-DEVD-fmk a specific inhibitor of the apoptosis in the cryopreservation medium of in vitro produced bovine embryos. In conclusion, addition of AA (0.1 mM) in slow-freezing medium improves the cryosurvival of in vitro produced bovine embryos, reduces intracellular reactive oxygen species levels and DNA fragmentation. DTT and z-DEVD-fmk treatments had no effect on post-thaw embryo survival.

RESUMO

CARRASCAL-TRIANA, Erly Luisana, D.Sc., Universidade Federal de Viçosa, setembro de 2016. **Efeito dos antioxidantes ácido ascórbico e ditiotreitol, ou do inibidor de caspase-3 durante a criopreservação de embriões bovinos produzidos in vitro.** Orientador: Ciro Alexandre Alves Torres.

Os experimentos descritos no presente trabalho foram realizados com o objetivo geral de avaliar se a adição dos antioxidantes ácido ascórbico (AA) e ditiotreitol (DTT), ou o inibidor da caspase-3 (z-DEVD-fmk), durante a criopreservação podem melhorar o criotolerância de embriões bovinos produzidos in vitro. Foram realizados cinco experimentos representados assim: experimento 1, 2 e 3 no capitulo 1 e, experimento 4 e 5 no capitulo 2. Para todos os experimentos, os complexos cumulus-oócitos foram obtidos a partir de ovários de vacas de abatedouros. Os oócitos foram maturados, fertilizados e cultivados in vitro até o dia 7. Blastocistos e blastocistos expandidos foram aleatoriamente submetidos ao congelamento com taxa controlada seguindo o equilíbrio de 10 min em meio de congelamento (Hepes-TALP acrescido de 1,5 M de etilenoglicol e 0,1 M de sacarose) com os tratamentos tal como descrito abaixo. No experimento 1, os embriões foram equilibrados no meio de congelamento contendo 0,0; 0,1; 0,3 ou 0,5 mM de AA. Os embriões em palhetas foram então colocados numa máquina de congelamento programável em -6,0 °C a -32 °C antes de ser mergulhado em nitrogênio líquido (-196 °C). Em seguida, os embriões foram descongelados e cultivados durante 72 h em meio SOF-BE1 suplementado com 10% (v/v) de soro fetal bovino a 38,5 °C numa atmosfera umidificada de 5% de CO_2, 5% O_2 e 90% N_2. Os embriões tratados com 0,1 mM de AA apresentaram maiores taxa de re-expansão às 24, 48 e 72 h e na taxa de eclosão às 72 h em comparação com os embriões do controle ($P<0,05$), assim, essa concentração foi considerada como ótima para os seguintes experimentos. Para o experimento 2 e 3, os embriões foram criopreservados em meio de congelamento contendo ou não 0,1 mM de AA, em seguida foram descongeladas e cultivadas durante 24 h. As concentrações intracelulares de espécies reativas de oxigénio foram reduzidos ($P<0,001$) pelo antioxidante AA (30,3 ± 2,4) em comparação com o controle (49,3 ± 1,9). Não houve efeito sobre o número total de células de blastocisto ($P>0,05$). Contudo, o AA 0,1 mM reduziu ($P<0,001$) a percentagem de células apoptóticas e a fragmentação do DNA comparado ao grupo controle. Os experimentos 4 e 5 foram conduzidos para avaliar os efeitos do DTT ou z-DEVD-fmk durante a criopreservação. Blastocistos e

blastocistos expandidos foram equilibrados em meio de congelamento contendo DTT (0, 50, 100 e 200 µM) ou z-DEVD-fmk (0, 50, 100 e 200 µM). Os embriões em palhetas foram colocados numa máquina de congelamento programável em -6,0 °C a -32 °C antes de ser mergulhado em nitrogênio líquido (-196 °C). Os embriões foram descongelados e cultivados em seguida durante 72 h. A taxas de re-expansão e eclosão foram registradas às 24, 48 e 72 h. Não houve efeito (P>0,05) do tratamento com DTT ou z-DEVD-fmk nas taxas de re-expansão e eclosão às 24, 48 ou 72 h pós-descongelamento. Este é o primeiro trabalho que utiliza os antioxidantes AA e DTT ou o inibidor específico da apoptose z-DEVD-fmk no meio de criopreservação de embriões bovinos produzidos in vitro. Em conclusão, a adição de AA (0,1 mM) no meio de congelamento lento melhora a crio-sobrevivência de embriões bovinos produzidos in vitro, reduz as concentrações intracelulares de espécies reativas de oxigénio e a fragmentação do DNA. Os tratamentos DTT e z-DEVD-fmk não apresentaram nenhum efeito sobre a sobrevivência dos embriões pós-descongelamento.

INTRODUCTION

Cryopreservation of embryos is a crucial step for the widespread and conservation of animal genetic resources. All embryos suffer considerable morphological and functional damage during cryopreservation but the extent of the injury as well as differences in survival and developmental rates may be highly variable depending on the species, developmental stage, if in vitro or in vivo produced (Pereira and Marques, 2008) or perhaps the most important, on the conditions of culture and cryopreservation protocols.

The process of freezing and thawing increases production of reactive oxygen species (ROS). The three major types of ROS are superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl ($OH^{\bullet}$), an increase in these reactive species that exceeds the ability of the antioxidant system to counteract it leads to an “oxidative stress state” (Agarwal et al., 2005), which, in turn, may affect original cell functions. Normally the pathologic effects are exerted by various mechanisms including lipid damage, protein synthesis inhibition and ATP depletion (Gupta et al., 2009). The oxidative injury may induce release of apoptogenic factors from cell mitochondria which eventually activate programmed cell death (Orrenius et al., 2007). This type of cell death may occur by two pathways: the extrinsic (receptor-mediated) and intrinsic (stress-mediated). In both pathways, initiator caspases (caspase-2, 8 and/or 10) activates other effector caspases (caspase-3, 7 and 6) resulting on blebbing of nuclear membrane, and chromatin condensation and fragmentation, shrinking of the cell, mitochondrial disintegration and formation of apoptotic bodies (budding off cellular portions) which ultimately leads to cell death (Xu et al., 2001; Danial and Korsmeyer, 2004; Hussein, 2005; Faustino et al., 2011). In order to minimize oxidative damages, improvement of the cryopreservation solutions used is needed. There are strong indications that injuries caused by lipid peroxidation may be reduced by the use of antioxidants (Lane et al., 2002).

Ascorbic acid (AA) is an antioxidant and it is involved in processes of hormone production, gonadal tissue remodeling and apoptosis by its ability to protect cells from free radicals (Luck et al., 1995). Granulosa, theca and luteal cells, oocyte and follicular fluid are exceptionally rich in AA (Luck et al., 1995; Behrman et al., 1996). Studies report the direct effects of AA as antioxidant on the efficiency of in

vitro and in vivo produced embryos of laboratory animals and domestic species. It was demonstrated that embryos treated with AA improves cleavage rates, blastocyst rates and total cell numbers per blastocyst, reduces apoptotic index and intracellular ROS levels and increases calving rates (Tarín and Trounson, 1993; Tatemoto et al., 2001; Lane et al., 2002; Wang et al., 2002; Jeong et al., 2006; Korhonen et al., 2012; Castillo-Martín et al., 2014a,b,c; Carrascal-Triana et al., 2015).

Another strong reducing agent is the Dithiothreitol (DTT), an antioxidant used to reduce disulfide bonds quantitatively and maintain monothiols in the reduced state (Cleland, 1964), it contains two thiol groups for sequential thiol-disulfide exchange reactions for direct scavenging of ROS (Rothwarf and Scheraga 1992). DTT protects cells from apoptosis by precluding the phosphorylation of protein tyrosine residues (Watson et al. 1996), reduces the thiol-oxidative damage caused by diamide in zygotes and blastocysts (Liu et al., 1999). Increases fertilization rate, the number of cells post-insemination and potential development of oocytes to blastocyst stage (Tarín et al., 1998) and blocks apoptosis induced by the heat-shock (De Castro e Paula and Hansen, 2008). Its action has not been yet tested in cryopreservation protocols.

It is known that cryopreservation may result in fragmentation of DNA even in the absence of cellular morphology deformation, and these changes can lead to delay in the development or cell death. The cell death can be controlled by a variety of inhibitors of caspase that directly interact with the protease. The caspase-3 inhibitor (z-DEVD-fmk), a specific inhibitor of group II caspases (ie., caspase-2, -3 and -7; Jousan et al., 2008) blocks the induction of apoptosis and DNA fragmentation caused by heat-shock in the oocyte and preimplantation embryos (Paula-Lopes and Hansen, 2002; Roth and Hansen, 2004; Jousan and Hansen, 2007). It is reasonable to suppose that the use of z-DEVD-fmk in freezing protocols may inhibit apoptosis, avoiding the damage and compromising subsequent developmental competence post-thawing of embryos.

The purpose of the current studies is achieving cryopreservation protocols capable of maintaining the integrity and survival of IVP bovine embryos after thawing. Thus, a review was done to describe the structure, biosynthesis, production and distribution of AA and its main biological functions as antioxidant on the reproductive activity. Five studies were conducted to determine whether treatments

with the antioxidants AA and DTT, or z-DEVD-fmk during cryopreservation might improve the cryotolerance of in vitro produced bovine embryos.

References

AGARWAL, A.; GUPTA, S.; SHARMA, R. K. (2005) Role of Oxidative Stress in Female Reproduction. **Reproductive Biology and Endocrinology**, v. 3, p. 28. doi:10.1186/1477-7827-3-28

BEHRMAN, H. R.; PRESTON, S. L.; ATEN, R. F.; RINAUDO, P.; ZREIK, T. G. (1996) Hormone Induction of Ascorbic Acid Transport in Immature Granulosa Cells. **Endocrinology**, v. 137, n. 10, p. 4316–4321.

CARRASCAL-TRIANA , E. L.; ZOLINI, A. M.; RUIZ, A.; PENITENTE-FILHO, J. M.; TORRES, C. A. A.; BLOCK, J. (2015) Post-Thaw Viability of Bovine Embryos Produced in vitro Following Treatment with Ascorbic Acid, Dithiothreitol, and Caspase-3 Inhibitor During Cryopreservation. **Reproduction, Fertility and Development**, v. 28(2), p. 146–147. doi: 10.1115/HT2013

CASTILLO-MARTÍN, M.; YESTE, M.; SOLER, A.; MORATÓ, R, S. B. (2014a) Comparative Effects of Adding β-Mercaptoethanol or L-Ascorbic Acid to Culture or Vitrification-Warming Media on IVF Porcine Embryos. **Reproduction, Fertility and Development**, v. 26, n. 6, p. 875–882.

CASTILLO-MARTÍN, M.; BONET, S.; MORATÓ, ROSER.; M.; YESTE. (2014b) Supplementing Culture and Vitrification-Warming Media with L-Ascorbic Acid Enhances Survival Rates and Redox Status Of IVP Porcine Blastocysts Via Induction Of GPX1 and SOD1 Expression. **Cryobiology,** v. 68, n. 3, p. 451–458. doi:10.1016/j.cryobiol.2014.03.001

CASTILLO-MARTÍN, M.; YESTE, M.; SOLER, A.; MORATÓ, ROSER; BONET, S. (2014c) Addition of L-Ascorbic Acid to Culture and Vitrification Media of IVF Porcine Blastocysts Improves Survival and Reduces HSPA1A Levels of Vitrified Embryos. **Reproduction, Fertility and Development**, v. 27, n. 7, p. 1115–1123. doi: 10.1071/RD14078

CLELAND, W. W. (1964) Dithiothreitol, a New Protective Reagent for SH Groups. **Biochemistry,** v.3 (4), p. 480–482. doi: 10.1021/bi00892a002

DANIAL, N.N.; KORSMEYER, S.J. (2004) Cell Death: Critical Control Points. **Cell,** v. 23, n. 116 (2) p. 205-19.

DE CASTRO E PAULA, L. A.; HANSEN, P. J. (2008) Modification of Actions of Heat Shock on Development and Apoptosis of Cultured Preimplantation Bovine Embryos by Oxygen Concentration and Dithiothreitol. **Molecular Reproduction and Development**, v.75, p. 1338–1350. doi:10.1002/mrd.20866

FAUSTINO, L. R.; CARVALHO, A. A.; DA SILVA, C. M. G.; FIGUEIREDO, J. R.; RODRIGUES A. P. R. (2011) Criopreservação de Tecido Ovariano: Limitações e Perspectivas para a Preservação da Fertilidade de Fêmeas. **Acta Scientiae Veterinariae**. v. 39, n. 3:972.

GUPTA, S.; MALHOTRA, N.; SHARMA, D.; CHANDRA, A.; ASHOK, A. (2009) Oxidative Stress and its Role in Female Infertility and Assisted Reproduction: Clinical Implications. **Royan Institue International Journal of Fertility and Sterility**, v. 2, n. 4, p. 147–164.

HUSSEIN M.R. (2005) Apoptosis in the Ovary: Molecular Mechanisms. **Human Reproduction Update**, v. 11 n. (2), p. 162-177. doi: 10.1093/humupd/dmi001

JEONG, Y. W.; PARK, S. W.; HOSSEIN, M. S.; KIM, S.; KIM, J. H.; LEE, S. H.; KANG, S. K.; LEE, B. C.; HWANG, W. S. (2006) Antiapoptotic and Embryotrophic Effects of Β-Tocopherol and L-Ascorbic Acid on Porcine Embryos Derived From in vitro Fertilization and Somatic Cell Nuclear Transfer. **Theriogenology**, v. 66, n. 9, p. 2104–2112.

JOUSAN, F. D.; HANSEN, P. J. (2007) Insulin-Like Growth Factor-I Promotes Resistance of Bovine Preimplantation Embryos to Heat Shock Through Actions Independent of its Anti-Apoptotic Actions Requiring PI3K Signaling.

Molecular Reproduction and Development, v.74, p. 189-196. doi: 10.1002/mrd.20527

KORHONEN, K.; JULKUNEN, H.; KANANEN, K.; BREDBACKA, P.; TIIRIKKA, T.; RÄTY, M.; VARTIA, K.; KAIMIO, I.; KONTINEN, A.; HALMEKYTÖ, M.; VILKKI, J.; PEIPPO, J.; LINDEBERG, H. (2012) The Effect Of Ascorbic Acid During Biopsy and Cryopreservation on Viability of Bovine Embryos Produced in vivo. **Theriogenology**, v. 77, n. 1, p. 201–205. doi: 10.1016/j.theriogenology.2011. 07.034

LANE, M.; MAYBACH, J.; GARDNER, D. (2002) Addition of Ascorbate During Cryopreservation Stimulates Subsequent Embryo Development. **Human Reproduction**, v. 17, n. 10, p. 2686–2693. doi: 10.1093/humrep/17.10.2686

LIU, L.; TRIMARCHI, J. R.; KEEF, D. L. (1999) Thiol Oxidation-Induced Embryonic Cell Death in Mice is Prevented by the Antioxidant Dithiothreitol. **Biology of Reproduction**, V.61, p. 1162–1169. doi: 10.1095/ biolreprod61.4.1162

LUCK, M. R.; JEYASEELAN, I.; SCHOLES, R. A. (1995) Ascorbic Acid and Fertility. **Biology of Reproduction**, v. 52, n. 2, p. 262–266. doi: 10.1095/biolreprod52.2.262

ORRENIUS, S.; GOGVADZE, V.; ANNU, B. Z. (2007) Mitochondrial Oxidative Stress: Implications for Cell Death. **Rev. Pharmacol. Toxicol**. v. 47, p. 143–83. doi: 10.1146/annurev.pharmtox.47.120505.105122

PAULA-LOPES, F. F.; HANSEN, P. J. (2002) Apoptosis is an Adaptive Response in Bovine Preimplantation Embryos That Facilitates Survival After Heat Shock. **Biochemical and Biophysical Research Communications**, v.295, p. 37-42. doi: 10.1016/S0006-291X(02)00619-8.

PEREIRA, R. M.; MARQUES, C. C. (2008) Animal oocyte and embryo cryopreservation. **Cell Tissue Banking** v. 9, p. 267–277.

ROTH, Z.; HANSEN, P.J. (2004) Involvement of Apoptosis in Disruption of Developmental Competence of Bovine Oocytes by Heat Shock During Maturation. **Biology of Reproduction,** v.71, p. 1898-1906.

ROTHWARF, D. M.; SCHERAGA, H. A. (1992) Equilibrium and Kinetic Constants for the Thiol-Disulfide Interchange Reaction Between Glutathione and Dithiothreitol. **Proc Natl Acad Sci U S A,** v.89, p.7944-7948. PMCID: PMC49831

TARÍN, J. J.; TROUNSON, A. O. (1993) Effects of Stimulation or Inhibition of Lipid Peroxidation on Freezing-Thawing of Mouse Embryos. **Biology of reproduction,** v. 49, n. 6, p. 1362–1368. PMID: 8286618

TATEMOTO, H.; OOTAKI, K.; SHIGETA, K.; MUTO, N. (2001) Enhancement of Developmental Competence After in vitro Fertilization of Porcine Oocytes by Treatment with Ascorbic Acid 2-O-Alpha-Glucoside During in vitro Maturation. **Biology of Reproduction**, v. 65, n. 6, p. 1800–1806,

XU, J.; CHEUNG, T.; CHAN, S. T.; PAK-CHUNG, M. D.; YEUNG, W. S. (2001) The Incidence of Cytoplasmic Fragmentation in Mouse Embryos in vitro is not Affected by Inhibition of Caspase Activity. **Fertility and Sterility**. v.75, n5, p. 986- 991.

WANG, X.; PH, D.; FALCONE, T.; ATTARAN, M. (2002) Vitamin C and Vitamin E Supplementation Reduce Oxidative Stress – Induced Embryo Toxicity and Improve the Blastocyst. **Fertility and Sterility**, v. 78, n. 6, p.1272-1277.

WATSON, R. W. G.; ROTSTEIN, O. D.; NATHENS, A. B.; ALAN, P. B.; Dackiw.;. Marshall, J. C. (1996) Thiol-Mediated Redox Regulation of Neutrophil Apoptosis. **Department of Surgery,** University of Toronto, Canada. V.120, p. 150–158.

CHAPTER 1

Ascorbic Acid: Implication on the reproductive activity (a review)

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Introduction

Ascorbic acid (AA) is a hydrosoluble vitamin, classified as an antioxidant. It is widely distributed in animal tissues and is present mainly in the pituitary, adrenal and gonads (ovaries and seminal plasma; Padh, 1991; Das et al., 1993). AA has three biological actions of particular relevance to reproduction, each dependent on its role as a reducing agent (Luck et al., 1995). It is essential to prevent or reduce the oxidation of biomolecules due to its ability to donate electrons, it is required for the biosynthesis of collagen, important for follicular growth, ovulation and corpus luteum formation, additionally, AA positively influences the process of steroidogenesis.

These biological actions play an important role in the protection to the cell including the prevention of mutations in DNA (Lindahl and Wood 1999), protection against lipid peroxidation (Kimura et al., 1992; Barja et al., 1994), the repair of oxidized amino acids for protein integrity maintenance (Barja et al., 1994; Cadenas et al., 1998), inhibiting apoptosis in bovine granulosa cells and murine cumulus-oocyte complexes (Tilly and Tilly, 1995 ; Eppig et al., 2000; Murray et al., 2001), expression of antioxidant enzymes, such as SOD1 and GPX1, which reduce intracellular levels of reactive oxygen species in porcine blastocysts (Castillo-Martín et al., 2014b) and reducing levels of heat shock protein 70 (HSPA1A) in vitrified embryos (Castillo-Martín et al., 2014c).

A great progress in this area of research has been achieved in recent decades, due to the interest of scientists in getting animal reproductive efficiency using antioxidants in production protocols. The purpose of this review is to describe the

structure, biosynthesis, production and distribution of AA and the main biological functions of this antioxidant on the reproductive activity.

Ascorbic Acid Structure and Biosynthesis

Ascorbic acid (AA) is a hydrosoluble vitamin derived from glucose metabolism. It is an unstable, easily oxidized acid and can be destroyed by excess of oxygen, alkali and high temperature (Iqbal et al., 2004). Its chemical composition is $C_6H_8O_6$, with a molecular weight of 176. The AA molecule contains several reactive hydroxyl groups, especially at the 2- and 3- positions allowing a variety of derivatives to be easily synthesized (Roomi et al., 2015).

In fish, amphibians and reptiles, the enzymes involved in AA biosynthesis are located mainly in the kidneys, while in mammals and bird species these enzymes are found mainly in the liver (Grollman and Lehninger, 1957; Chatterjee et al., 1961). However, species such as teleost fish, some passeriform birds, guinea pigs, and some primates like humans, have lost the ability to synthesize AA (Grollman and Lehninger, 1957; Linster and Van Schaftingen, 2007) due to the loss of the terminal enzyme in their biosynthetic pathway, L-gulonolactone oxidase. They therefore require a dietary supply of the vitamin, mainly synthesized by plants or fruits.

Interestingly, very different pathways have evolved for AA biosynthesis in animals, plants and yeasts (Arrigoni and Tullio, 2002). In animals (Figure 1), pathway starts with D-glucose-1-phosphate, which is activated by binding of a nucleotide (Uridine diphosphate-UDP) and is catalyzed by the enzyme UDP-glucose pyrophosphorylase. The UDP-glucose then undergoes oxidation to form UDP-glucuronate, which is catalyzed by UDP-glucose dehydrogenase enzyme. D-glucuronate formed from the hydrolysis UDP-glucuronate is the precursor of L-ascorbic acid. On this pathway, D-glucuronate is reduced to the sugar L-gulonate by glucuronate reductase. Subsequently, L-Gulonate is converted to L-gulono-1,4-lactone by a gulonolactonase and the last step is the oxidation of L-gulono-1,4-lactone to L-ascorbic acid by L-gulonolactone oxidase (Linster and Van Schaftingen, 2007).

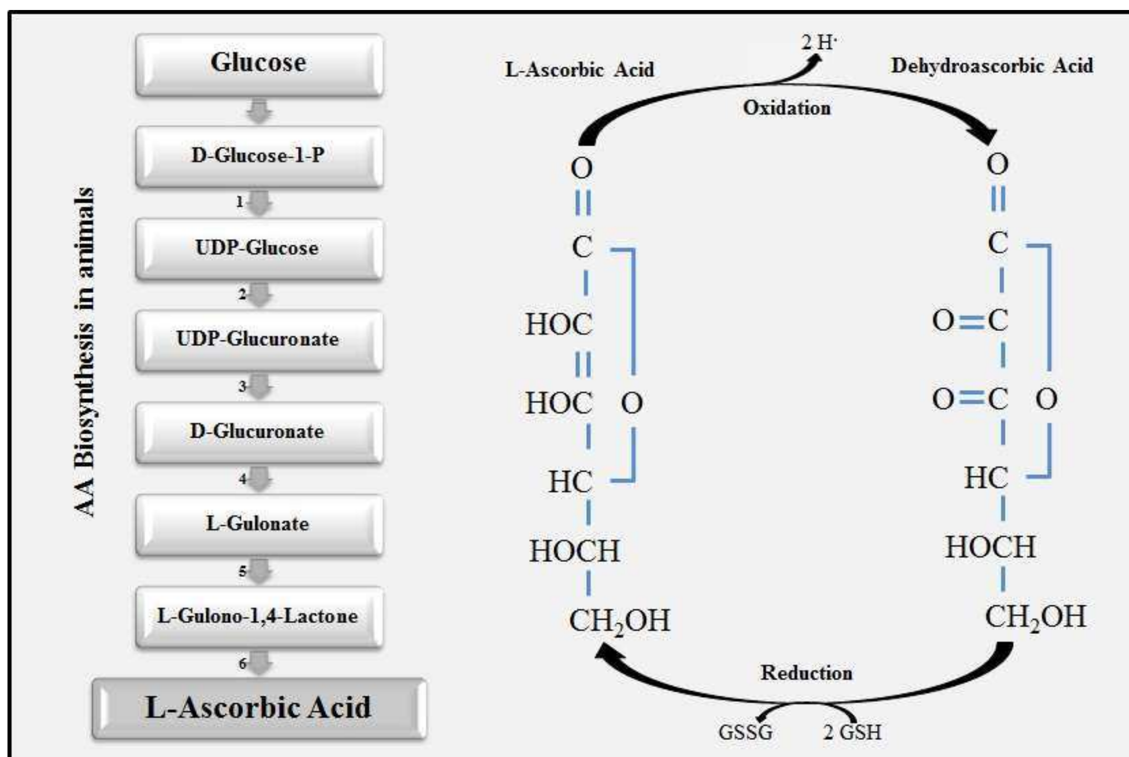

Figure 1. Ascorbic acid structure, biosynthesis and oxidation-reduction reaction. The reactions are catalyzed by the following enzymes: 1, UDP-glucose pyrophosphorylase; 2, UDP-glucose dehydrogenase; 3, Glucuronate-1-phosphate uridylyltransferase and Glucurono kinase; 4, Glucuronate reductase; 5, gulonolactonase; 6, L-gulonolactone oxidase.

AA is easily oxidized and converted to dehydroascorbic acid (Figure 1). AA may lose the hydrogen ions attached to one of its two ionizable groups located at carbons 2′ and 3′, generating ascorbate monoanion or dianion, dehydroascorbic acid (Tolbert et al., 1975; Du et al., 2012; Figueroa-Méndez and Rivas-Arancibia, 2015). In many cells of different species, including mammals, the conversion is reversible to some degree, and is catalyzed by a different enzyme, dehydroascorbate reductase. That enzyme uses reduced glutathione (GSH) as a cosubstrate, and the products are AA and oxidized glutathione (GSSG) (Englard and Seifter 1986; Bánhegyi et al., 1997).

In vitro studies have found that dehydroascorbic acid enters the cell via nonspecific, low-affinity, high-capacity glucose transporters (GLUTs – hexose transporters) and is reduced intracellularly to L- ascorbic acid. AA is also directly taken up by the cells through specific, high-affinity, low-capacity sodium ascorbate transporters (SVCTs – sodium-ascorbate cotransporter). SVCTs are expressed in the cells of spleen, brain, eye, pancreas, testis, ovary, adrenal gland and lung (Agus et al., 1997; Wilson, 2005; Roomi et al., 2015).

Ascorbic Acid Production and Distribution

Some mammals synthesize AA, however the concentrations found in the body sometimes are not sufficient to nutritional and reproductive needs of the animals. AA production is variable between species (Table 1) and depend on circumstances, in conditions of stress or infection, the synthesis of AA can be easily multiplied (Holford, 1997).

Table 1. Ascorbic acid produced per day by different animal species (equivalent for body weight of 10 Kg).

Animal species	AA production
Goat	530-3090 mg
Rat	636-3230 mg
Rabbit	359-3676 mg
Cow	255-298 mg
Mouse	547-4473 mg
Sheep	403 mg
Cat	78-651 mg

Source: Adapted from Holford (1997)
*adjusted for metabolic weight $P^{0,75}$

Plasma AA concentrations are maintained between 10 and 160 mM in man and highest amounts were found in adrenal glands (550 mg/kg), brain (140 mg/kg) and liver (125 mg/kg), followed by lungs (70 mg/kg), kidneys (55 mg/kg), heart (55 mg/kg), skeletal muscle (35 mg/kg) and skin (30 mg/kg), and low levels in adipose tissue (10 mg/kg) and blood (9 mg/kg); (Brown and Jones, 1996; Fuchs and Podda, 1997; Richelle et al., 2006)

The ovarian tissue, and especially the corpus luteum, are exceptionally rich in AA (up to 90 mg/100 g in cow ovaries), the follicular fluid itself contain 0.8-3.0 mg AA in 100 mL of fluid (Lutwak-Mann, 1954). AA is concentrated in granulosa cells, theca cells, luteal cells, and the oocyte, and its uptake is hormonally regulated (Luck et al., 1995; Behrman et al., 1996).

Biological Functions of Ascorbic Acid

AA has three biological functions of particular relevance to reproduction, each dependent on its role as a reducing agent: It is required to 1) prevent or reduce

the oxidation of biomolecules; 2) for the biosynthesis of collagen and 3) for the steroidogenesis process (Luck et al., 1995).

Role of ascorbic acid as antioxidant

Oxidation and reduction (Redox) reactions represent the transfer of electrons from an electron donor (reduction agent) to an electron acceptor (oxidation agent). The cellular redox environment is a balance between the production of reactive oxygen species (ROS), reactive nitrogen species, and their removal by antioxidant enzymes and small molecular-weight antioxidants (Sarsour et al., 2009). The three major types of ROS are: superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl ($OH^{\bullet}$), an increase in those reactive species that exceeds the ability of the antioxidant system to counteract the increase of ROS leads to an "oxidative stress state" (Agarwal et al., 2005). Oxygen radicals and ROS play both a physiologic and pathologic role in the female reproductive tract. Normally the pathologic effects are exerted by various mechanisms including lipid damage, inhibition of protein synthesis, and depletion of ATP (Gupta et al., 2009). ROS affects multiple physiological processes from oocyte maturation to fertilization, embryo development and pregnancy (Agarwal et al., 2005). The propagation of oxidative stress can be prevented by AA antioxidant. AA stops the peroxidative process due to its ability to donate electrons from both the second and third carbon (Padayatty et al., 2003; Mahfouz and Kummerow, 2004). AA also helps recycle oxidized vitamin E and glutathione (Chan, 1993; Venereo Gutiérrez, 2002; Agarwal et al., 2005) and it's a cofactor in the biosynthesis of L-carnitine a molecule required for the oxidation of fatty acids (Oguntibeju, 2008). L-carnitine has been used strategically to reduce embryo lipid content and improve cryotolerance of embryos (Ruiz et al., 2015; Zolini et al., 2015).

Ascorbic acid in embryo production

Rizo et al. (2003) compare bovine oocyte maturation, fertilization and embryo culture in vivo and in vitro and demonstrated that the origin of the oocyte is the main factor affecting blastocyst yield while the post-fertilization culture environment is critical in determining blastocyst quality, measured in terms of cryotolerance. In vitro produced embryos are less cryotolerants compared to those in

vivo produced due to differences in the biochemical composition especially the amount of lipids within the cytoplasm, and an increased susceptibility to low temperatures or an increase in cellular damage (Kaidi et al., 2001; Lonergan et al., 2003; Seidel, 2006). Moreover, the embryonic genome activation, embryonic compaction and hatching are processes that demand greater amounts of energy (Gott et al., 1990). The embryo produces energy via ATP generation through mitochondrial oxidative phosphorylation and glycolysis (Agarwal et al., 2014). Processes of developing embryo usually generate ROS. In addition, the reproductive technology procedures (techniques and conditions employed) contribute to the higher levels of oxidative stress. The accumulation of lipids and consequent increase in lipid peroxidation caused by ROS are largely responsible for cryotolerance reduction and lower development of IVP embryos (Nedambale et al., 2006). To reduce damage induced by oxidative stress, antioxidant AA has often been used as a supplement in embryonic development and cryopreservation systems of different species. Key findings of studies using antioxidant AA in medium are summarized in Table 2.

Table 2. Studies involving antioxidant AA in embryo production of different animal species.

References	Biotechnical	Species	AA Conc	Results
Tarín and Trounson, 1993	IVC	Mouse	62.5 µM	AA increased embryo survival to the freezing-thawing stress, blastocysts on Day 5 and implantations rates.
Tatemoto et al., 2001	IVM	Porcine	250 µM	AA2G protects oocytes against oxidative stress and post-cleavage development to the blastocyst stage.
Wang et al., 2002	IVC	Mouse	50 µM	AA is effective to reverse ROS-induced embryo toxicity and improve embryo development.
Lane et al., 2002	Slow-freezing; Vitrification	Mouse	0.1 mM	AA reduced the levels of hydrogen peroxide, increases inner cell mass development and rates of metabolism.
Tao et al., 2004	IVM	Porcine	250 µM	AA promotes the development of denuded oocytes from MI to MII and prevents cumulus cell DNA fragmentation.
Dalvit et al., 2005	IVM	Bovine	5 mM	AA maintained the antioxidant capacity of the alpha-tocopherol incorporated to cumulus–oocyte complexes membranes.

Jeong et al., 2006	IVC	Porcine	100 μM	AA improves blastocyst formation, number of total cells and reduces the number of apoptotic blastomeres.
Hossein et al., 2007	IVC	Porcine	100 μM	AA supplements at 0 and 96 h of culture improves embryo development and quality.
Ferro et al., 2009	IVC	Bovine	10 μg/mL	The addition of AA to BARC culture medium during 5 to 72 h post-insemination increased in vitro embryo production.
Córdova et al., 2010	IVM	Bovine	100 μg/mL	AA plus insulin-transferrin-selenium supplementation during the first 12 h of IVM improves cytoplasm maturation and the developmental of embryos.
Korhonen et al., 2012	Biopsy; Slow-freezing	Bovine	0.1 mM	If biopsy and freezing are used prior to embryo transfer, addition of AA into the media could result in an appreciable increase in calving rate.
Kere et al., 2013	IVM; IVC	Porcine	50 μg/mL	AA supplementation improves blastocyst rates and total cell numbers, reduces apoptotic indices.
Castillo-Martín et al., 2014a	IVC; Vitrification	Porcine	100 μM	ROS levels and survival rates after vitrification–warming were significantly improved in embryos cultured with AA.
Castillo-Martín et al., 2014b	IVC; Vitrification	Porcine	100 μM	AA increases gene expression of antioxidant enzymes SOD1 and GPX, improve redox balance and embryo cryosurvival.
Castillo-Martín et al., 2014c	IVC; Vitrification	Porcine	100 μM	AA enhances survival rates of blastocysts, suggesting a relationship with HSPA1A expression.
Carrascal-Triana et al., 2015	Slow-freezing	Bovine	0.1 mM	Embryos frozen in freezing medium containing AA had increased re-expansion and hatching rates at 24, 48 and 72 h post-thaw.

AA: ascorbic acid; AA2G: ascorbic acid 2-O-α-glucoside; IVM: in vitro Maturation; IVC: in vitro Culture; BARC: Betsville Agriculture Research Center medium.

Studies report the direct effects of AA as antioxidant on the efficiency of in vitro and in vivo produced embryos in laboratory animals and domestic species. It was demonstrated that embryos treated with AA improves cleavage rates, blastocyst rates and total cell numbers per blastocyst, reduces apoptotic index and intracellular ROS levels and increases calving rates. However, embryo-trophic effects continue to be discussed because it has not been defined the ideal concentration and duration of

AA supplementation in the different stages of embryos production. Higher concentration than 200 μM produces toxicity in mouse embryos (Wang et al., 2002) and bovine oocytes (Dalvit et al., 2005) and does not improve blastocyst formation nor the blastocyst quality in porcine (Hossein et al., 2007). Tatemoto et al. (2001) found that higher concentrations (500 and 750 μM) of ascorbic acid 2-O-α-glucoside (AA-2G), a stable AA derivative, showed no effect on the induction of oocyte maturation, however, they suggested the addition of 250 μM AA-2G for supporting cytoplasmic maturation in porcine oocytes. Years later, Tao et al. (2004) demonstrated an increased atypical oocyte percentage when supplemented AA at 250 μM in cumulus-enclosed oocytes and 750 μM in denuded oocytes.

Ascorbic acid in preantral follicles

The development of in vitro culture systems that allow preantral follicles growth could revolutionize in vitro embryos production due to the thousands of oocytes present in the ovary. Excellent results have been obtained in embryos production of different species (Porcine: Wu et al., 2001), (Bubaline: Gupta et al., 2008), (Caprine: Saraiva et al., 2010; Magalhães et al., 2011), (Ovine: Arunakumari et al., 2010; Luz et al., 2012), (Bovine: McLaughlin et al., 2010), (Canine: Serafim et al., 2010) and (Human: Telfer et al., 2008). The birth of mouse oocytes from primordial follicles represent the highest advance made to the present (O'brien, 2003).

The continuous improvement of the culture medium is a crucial point for the efficiency of the manipulation of oocytes enclosed in preantral follicles. In the search for an ideal system, substances and intraovarian factors have been studied, among which can mention the AA, which has shown itself capable of maintaining follicular integrity through regulation and remodeling of the extracellular matrix and reduced apoptosis rates in cows (Thomas et al., 2001) and rats (Murray et al., 2001). The use of ascorbic acid provides protection to tissues against ROS molecules and metal-oxygen that can cause damage to DNA, proteins, carbohydrates, lipids and biological membranes (Frei et al., 1989).

Rossetto et al. (2009) evaluated the effects of AA and its interaction with FSH on the morphology, activation and in vitro growth of in-situ caprine preantral follicles and the results showed that after 14 days of culture, medium supplemented

with 50 μg/mL of AA alone or combined with FSH promoted higher rates of follicular survival, increased oocyte and follicular diameter and maintained follicular integrity. Similar results were demonstrated by Silva et al. (2011), the AA improved the survival and in vitro development of isolated caprine preantral follicles, and increased the production of the matrix metalloproteinase-9 (MMP-9) and their tissue inhibitor-2 (TIMP-2) by the cultured follicles, which implicates the effect of this vitamin in regulation of collagen on the basement membrane. The addition of AA at a concentration of 50 μg/mL was also studied in cattle by stimulating the activation, growth and viability of in vitro cultured primordial follicles (Andrade et al., 2012) and in in vitro culture system for equine ovarian tissue collected from a slaughterhouse (Gomes et al., 2015).

Vitrification is an important tool for preservation of follicular morphology and viability over long periods, however, this process causes damage to the follicular structure due to formation of ROS that cause lipid peroxidation (Frei et al., 1989). In order to minimize oxidative stress Melo et al. (2011) demonstrated that adding AA to the vitrification solution preserved the follicular morphology in ovine ovarian tissue but did not improve the viability or developmental capacity of the follicles. Carrascal-Triana et al. (2012) indicated that antioxidant AA improves survival rates and maintains the morphological integrity during the vitrification of bovine preantral follicles.

Role of ascorbic acid in the biosynthesis of collagen

One important function of AA is in the formation and maintenance of collagens, which are found in skin, ligaments, cartilages, vertebral discs, joint linings, capillary walls, bones, teeth (Iqbal et al., 2004), the extracellular matrix and basement membrane (Du et al., 2012). Type I collagen is the major structural protein in the interstitial extracellular matrix, while type IV is prevalent in basement membrane (Egeblad et al., 2010). In the ovary, the granulosa cell layer of each ovarian follicle is surrounded by a follicular basement membrane, which separates the follicles of the stromal elements or theca cells around them (Van Wezel and Rodgers, 1996).

For the type IV collagen synthesis is necessary the hydroxylation of proline and lysine amino acids forming the hydroxyproline and hydroxylysine. In this

reaction, AA is a cofactor for the enzyme prolyl hydroxylase, which catalyzes the hydroxylation, reduces the iron and facilitates the release of the enzyme (Myllylä et al., 1978). Collagen subunits are formed with fibroblasts as procollagen, which are excreted into extracelular spaces (Iqbal et al., 2004). AA is required to export the procollagen molecules out of the cell. The final structure of the collagen is formed after pieces of the procollagen are enzymatically cleaved (Gaby and Singh, 1991).

Adequate turnover of components of the extracellular matrix of the follicle is essential for normal follicle growth, follicle repair after ovulation (Himeno et al., 1984) and subsequent development of the corpus luteum (Luck and Zhao, 1993). Silva et al. (2011) demonstrated the importance of AA on the viability, growth, antrum formation and regulation of the enzymes MMP-9 and TIMP-2 involved in remodeling of the extracellular matrix of caprine preantral follicles after long-term culture in vitro. These results are in agreement with other previous studies in vitro, which have demonstrated promotion of follicle integrity and survival by the presence of AA in cultured mouse and bovine follicles (Murray et al., 2001; Thomas et al., 2001).

Role of Ascorbic Acid in the Steroidogenesis

The adrenal glands are composed of the cortex and medulla regions. The adrenal cortex consists of three zones that secrete aldosterone, cortisol and adrenal androgens. The adrenal medulla is functionally related to the sympathetic nervous system and secretes the catecholamines, epinephrine and norepinephrine (Rosol et al., 2001).

The concentration of AA in the adrenal glands is among the highest in the body. Interestingly, both the adrenal cortex and the medulla accumulate such high levels of AA (Patak et al., 2004). The AA function in the adrenal medulla can be conceptualized in three general areas: as a cofactor for dopamine β-hydroxylase in the conversion of dopamine to norepinephrine; as a cofactor for an amidation enzyme, AA may donate electrons for synthesis of other hormones, proteins, or peptides in chromaffin cells; and other function as a secreted neuromodulator (Levine and Morita, 1985). Luck and Jungclas (1987) have obtained evidence for the involvement of AA and catecholamines on bovine granulosa cells in the regulation of luteal oxytocin secretion. Oxytocin inhibits LH-stimulated prostacyclin production in

luteal cells of cows. Oxytocin may induce the release of PGF-2 alpha or lipo-oxygenase products from the ovary (Fuchs, 1988). The magnitude of the catecholamine effect strongly suggests a neuroendocrine involvement in the regulation of ovarian peptide secretion, although whether this may be through circulating catecholamines or through those derived from the sympathetic nerves which directly supply the ovary is not yet clear.

In adrenal cortical cells a major function of AA is to preserve cytochrome P450 levels that play important roles in the synthesis of steroid hormones (Hornsby, 1985). Particularly, the cytochrome P450 11B1 (11b-hydroxylase) is subject to destruction or inactivation through interaction with 11b-hydroxylated steroids, ie. the enzyme products, which act as pseudosubstrates. Experiments on fetal human adrenocortical cells in culture demonstrated the protective effects of AA during cortisol-induced loss of 11b-hydroxylase activity (Hornsby, 1980). On the other hand, Pintauro and Bergan (1982) support a possible regulatory role of AA on the conversion of pregnenolone to delta-4 and delta-5 steroids (Progesterone and dehydroepiandrosterone, respectively) with the addition of 0.5 mM AA.

References

AGARWAL, A.; DURAIRAJANAYAGAM, D.; DU PLESSIS, S. S. (2014) Utility of Antioxidants During Assisted Reproductive Techniques: An Evidence Based Review. **Reproductive Biology and Endocrinology**, v. 12, p. 112. doi:10.1186/1477-7827-12-112

AGARWAL, A.; GUPTA, S.; SHARMA, R. K. (2005) Role of Oxidative Stress in Female Reproduction. **Reproductive Biology and Endocrinology**, v. 3, p. 28. doi:10.1186/1477-7827-3-28

AGUS, D. B.; GAMBHIR, S. S.; PARDRIDGE, W. M.; SPIELHOLZ, C.; BASELGA, J.; VERA, J. C.; GOLDE, D. W. (1997) Vitamin C Crosses the Blood-Brain Barrier in the Oxidized Form Through the Glucose Transporters. **The Journal of clinical investigation**, v. 100, n. 11, p. 2842–2848. doi: 10.1172/JCI119832

ANDRADE, E. R.; VAN DEN HURK, R.; LISBOA, L. A.; HERTEL, M. F.; MELO-STERZA, F. A.; MORENO, K.; BRACARENSE, ANA PAULA F. R. L.; LANDIM-ALVARENGA, F. C.; SENEDA, M. M.; ALFIERI, A. A. (2012) Effects of Ascorbic Acid on in vitro Culture of Bovine Preantral Follicles. **Zygote** (Cambridge University, England), v. 20, n. 4, p. 379–388. doi:10.1017/S0967199412000056

ARRIGONI, O.; DE TULLIO, M. C. (2002) Ascorbic Acid: Much More Than Just an Antioxidant. **Biochim Biophys Acta.** v. 15, 1569(1-3):1-9

ARUNAKUMARI, G.; SHANMUGASUNDARAM, N.; RAO, V. H. (2010) Development of Morulae from the Oocytes of Cultured Sheep Preantral Follicles. **Theriogenology**, v. 74, n. 5, p. 884–894. doi.10.1016/j.theriogenology.2010.04.013

BÁNHEGYI, G.; BRAUN, L.; CSALA, M.; PUSKÁS, F.; MANDL, J. (1997) Ascorbate Metabolism and its Regulation in Animals. **Free Radical Biology & Medicine**, v. 23, n. 5, p. 793–803. doi:10.1016/S0891-5849(97)00062-2

BARJA, G.; LÓPEZ-TORRES, M.; PÉREZ-CAMPO, R.; ROJAS, C.; CADENAS, S.; PRAT, J.; PAMPLONA, R. (1994) Dietary Vitamin C Decreases Endogenous Protein Oxidative Damage, Malondialdehyde, and Lipid Peroxidation and Maintains Fatty Acid Unsaturation in the Guinea Pig Liver. **Biology and Medicine,** v. 17, n. 2, p. 105–115. doi: 0891-5849(94)E0026-F

BEHRMAN, H. R.; PRESTON, S. L.; ATEN, R. F.; RINAUDO, P.; ZREIK, T. G. (1996) Hormone Induction of Ascorbic Acid Transport in Immature Granulosa Cells. **Endocrinology**, v. 137, n. 10, p. 4316–4321. doi:10.1210/endo.137.10.8828491

BROWN, L. A. S., AND JONES, D. P. (1996) The biology of ascorbic acid. **In Handbook of Antioxidants**, eds. E. Cadenas and L. Packer, p. 117–154.

CADENAS, S.; ROJAS, C.; BARJA, G. (1998) Endotoxin Increases Oxidative Injury to Proteins in Guinea Pig Liver: Protection by Dietary Vitamin C. **Pharmacology & Toxicology**, v. 82, n. 1, p. 11–8. PMID: 9527640

CARRASCAL-TRIANA, E. L.; JIMENEZ, C. R.; AZEVEDO, J. L.; SILVEIRA, R. G.; PENITENTE FILHO, J. M.; TORRES, C. A. A. (2012) Association of Cryoprotectants and Ascorbic Acid to Cool and Vitrify Preantral Bovine Follicles -. IV **International Symposium on Animal Biology of Reproduction (ISABR)**.

CARRASCAL-TRIANA , E. L.; ZOLINI, A. M.; RUIZ, A.; PENITENTE-FILHO, J. M.; TORRES, C. A. A.; BLOCK, J. (2015) Post-Thaw Viability of Bovine Embryos Produced in vitro Following Treatment with Ascorbic Acid, Dithiothreitol, and Caspase-3 Inhibitor During Cryopreservation. **Reproduction, Fertility and Development**, v. 28(2), p. 146–147. doi: 10.1115/HT2013

CASTILLO-MARTÍN, M.; YESTE, M.; SOLER, A.; MORATÓ, R, S. B. (2014a) Comparative Effects of Adding β-Mercaptoethanol or L-Ascorbic Acid to Culture or Vitrification-Warming Media on IVF Porcine Embryos. **Reproduction, Fertility and Development**, v. 26, n. 6, p. 875–882. doi:10.1071/RD13116

CASTILLO-MARTÍN, M.; BONET, S.; MORATÓ, ROSER.; M.; YESTE. (2014b) Supplementing Culture and Vitrification-Warming Media with L-Ascorbic Acid Enhances Survival Rates and Redox Status Of IVP Porcine Blastocysts Via Induction Of GPX1 and SOD1 Expression. **Cryobiology,** v. 68, n. 3, p. 451–458. doi:10.1016/j.cryobiol.2014.03.001

CASTILLO-MARTÍN, M.; YESTE, M.; SOLER, A.; MORATÓ, ROSER; BONET, S. (2014c) Addition of L-Ascorbic Acid to Culture and Vitrification Media of IVF Porcine Blastocysts Improves Survival and Reduces HSPA1A Levels of Vitrified Embryos. **Reproduction, Fertility and Development**, v. 27, n. 7, p. 1115–1123. doi: 10.1071/RD14078

CHAN, A. C. (1993) Partners in Defense, Vitamin E and Vitamin C. Canadian Journal of Physiology and Pharmacology, v. 71, n. 9, p. 725–731. doi:10.1139/y93-109

CHATTERJEE, I. B.; KAR, N. C.; GHOSH, N. C.; GUHA, B. C. (1961) Aspects of Ascorbic Acid Biosynthesis in Animals. **Annals of the New York Academy of Sciences**, v. 92, n. 1, p. 36–56. doi:10.1111/j.1749-6632.1961.tb46105.x

CÓRDOVA, B.; MORATÓ, R.; IZQUIERDO, D.; PARAMIO, T.; MOGAS, T. (2010) Effect of the Addition of Insulin-Transferrin-Selenium and/or L-Ascorbic Acid to the in vitro Maturation of Prepubertal Bovine Oocytes on Cytoplasmic Maturation and Embryo Development. **Theriogenology**, v. 74, n. 8, p. 1341–1348. doi:10.1016/j.theriogenology.2010.06.003

DALVIT, G.; LLANES, S. P.; DESCALZO, A.; INSANI, M.; BECONI, M.; CETICA, P. (2005) Effect of Alpha-Tocopherol and Ascorbic Acid on Bovine Oocyte in vitro Maturation. **Reproduction in Domestic Animals,** v. 40, n. 2, p. 93–97. doi:10.1111/j.1439-0531.2004.00522.x

DAS, P. C.; DAS, K. P.; BAGCHI, K.; DEY, C. D. (1993) Evaluation of Tissue Ascorbic Acid Status in Different Hormonal States of Female Rat. **Life Sci** v. 52, p. 1493–1498.

DEVINE, P. J.; PERREAULT, S. D.; LUDERER, U. (2012) Roles of Reactive Oxygen Species and Antioxidants in Ovarian Toxicity. **Biology of Reproduction,** v. 86, n. 2, p. 27. doi:10.1095/biolreprod.111.095224

DU, J.; CULLEN, J. J.; BUETTNER, G. R. (2012) Ascorbic Acid: Chemistry, Biology and the Treatment of Cancer. **Biochimica et Biophysica Acta**, v. 1826, n. 2, p. 443–457. doi:10.1016/j.bbcan.2012.06.003

EGEBLAD, M.; RASCH, M. G.; WEAVER, V. M. (2010) Dynamic Interplay Between the Collagen Scaffold and Tumor Evolution. **Current Opinion in Cell Biology**, v. 22, p. 697–706. doi: 10.1016/j.ceb.2010.08.015

ENGLARD, S.; SEIFTER, S. (1986) The Biochemical Functions of Ascorbic Acid. **Annual Review of Nutrition,** v. 6, p. 365–406. doi:10.1146/annurev.nutr.6.1.365

EPPIG, J. J.; HOSOE, M.; O'BRIEN, M. J.; PENDOLA, F. M.; REQUENA, A.; WATANABE, S. (2000) Conditions That Affect Acquisition of Developmental Competence by Mouse Oocytes in vitro: FSH, Insulin, Glucose and Ascorbic Acid. **Molecular and Cellular Endocrinology**, v. 163, n. 1–2, p. 109–116,. doi: 10.1016/S0303-7207(99)00247-6

FERRO, A. C.; SILVA, C.; CALEGARI, R. S. (2009) Produção de Embriões in vitro em Meio de Cultura com Ácido Ascórbico. **Vet. e Zootec**, v. 16, n. 1, p. 80–88.

FIGUEROA-MÉNDEZ, R.; RIVAS-ARANCIBIA, S. (2015) Vitamin C in Health and Disease: Its Role in the Metabolism of Cells and Redox State in the Brain. **Frontiers in Physiology**, v. 6, p. 1–11, doi 10.3389/fphys.2015.00397

FREI, B.; ENGLAND, L.; AMES, B. N. (1989) Ascorbate is an Outstanding Antioxidant in Human Blood Plasma**. Proceedings of the National Academy of Sciences of the United States of America**, v. 86, n. 16, p. 6377–6381. PMID: 2762330

FUCHS, A. R. (1988) Oxytocin and Ovarian Function. **Journal of Reproduction and Fertility,** v.36, p.39-47.

FUCHS, J.; PODDA, M. (1997) Vitamin C in Cutaneous Biology. In L Packer, J Fuchs (eds), Vitamin C in Health and Disease, **Marcel Dekker Inc**., New York, p. 333-340.

GABY, S.K.; SINGH, V. N. (1991) "Vitamin C," Vitamin intake and health: A Scientific Review, Gaby, S.K., Bendich, A., Singh, V. and Machlin, L. (eds.). **Marcel Dekker Inc**., New York, p. 103-1043.

GOMES, R. G.; LISBOA, L. A.; SILVA, C. B.; MAX, M. C.; MARINO, P. C.; OLIVEIRA, R. L.; GONZÁLEZ, S. M.; BARREIROS, T. R. R.; MARINHO, L. S. R.; SENEDA, M. M. (2015) Improvement of Development of Equine Preantral Follicles after 6 Days of in vitro Culture with Ascorbic Acid Supplementation. **Theriogenology**, v. 84, p. 750–755, doi: 10.1016/j.theriogenology.2015.05.006

GOTT, A. L.; HARDY, K.; WINSTON, R. M.; LEESE, H. J. (1990) Non-Invasive Measurement of Pyruvate and Glucose Uptake and Lactate Production by Single Human Preimplantation Embryos. **Human Reproduction** (Oxford, England), v. 5, n. 1, p. 104–108. PMID: 2324239

GROLLMAN, A. P.; LEHNINGER, A. L. (1957) Enzymic Synthesis of L-Ascorbic Acid in Different Animal Species. **Archives of Biochemistry and Biophysics**, v. 69, p. 458–467. doi:10.1016/0003-9861(57)90510-6

GUPTA, P. S. P.; RAMESH, H. S.; MANJUNATHA, B. M.; NANDI, S.; RAVINDRA, J. P. (2008) Production of Buffalo Embryos Using Oocytes From in vitro Grown Preantral Follicles. **Zygote** (Cambridge, England), v. 16, n. 1, p. 57–63. doi:10.1017/S096719940700442X

GUPTA, S.; MALHOTRA, N.; SHARMA, D.; CHANDRA, A.; ASHOK, A. (2009) Oxidative Stress and its Role in Female Infertility and Assisted Reproduction: Clinical Implications. **Royan Institue International Journal of Fertility and Sterility**, v. 2, n. 4, p. 147–164.

HIMENO, N.; KAWAMURA, N.; OKAMURA, H.; MORI, T.; FUKUMOTO, M.; MIDORIKAWA, O. (1984) Collagen Synthetic Activity in Rabbit Ovary During Ovulation and its Blockage by Indomethacin. **Nihon Sanka Fujinka Gakkai Zasshi**, v. 36, n. 10, p. 1930. PMID: 6094689

HOLFORD, I. C. R. (1997) Soil Phosphorus: its Measurement, and its Uptake by Plants. **Australian Journal of Soil Research**, v. 35, n. 2, p. 227. doi: 10.1071/SR96047

HORNSBY, P. J. (1980) Regulation of Cytochrome P-450-Supported 11β-Hydroxylation of Deoxycortisol by Steroids, Oxygen, and Antioxidants in Adrenocortical Cell Cultures. **Journal of Biological Chemistry**, v. 255, n. 9, p. 4020–4027.

HORNSBY, P. J.; HARRIS, S. E.; ALDERN, K. A. (1985) The Role of Ascorbic Acid in the Function of the Adrenal Cortex: Studies in Adrenocortical Cells in Culture. **Endocrinology,** v. 117, n. 3, p. 1264–1271. doi:10.1210/endo-117-3-126

HOSSEIN, M. S.; HASHEM, M. A.; JEONG, Y. W.; LEE, M. S.; KIM, S.; KIM, J. H.; KOO, O. J.; PARK, S. M.; LEE, E. G.; PARK, S. W.; KANG, S. K.; LEE, B. C.; HWANG, W. S. (2007) Temporal Effects of A-Tocopherol and L-Ascorbic Acid on in vitro Fertilized Porcine Embryo Development. **Animal Reproduction Science**, v. 100, n. 1–2, p. 107–117. doi:10.1016/j.anireprosci.2006.06.013

IQBAL, K.; KHAN, A.; ALI KHAN KHATTAK, M. M. (2004) Biological Significance of Ascorbic Acid (Vitamin C) in Human Health - A Review. **Pakistan Journal of Nutrition**, v. 3, n. 1, p. 5–13. doi:10.3923/pjn.2004.5.13

JEONG, Y. W.; PARK, S. W.; HOSSEIN, M. S.; KIM, S.; KIM, J. H.; LEE, S. H.; KANG, S. K.; LEE, B. C.; HWANG, W. S. (2006). Antiapoptotic and Embryotrophic Effects of Β-Tocopherol and L-Ascorbic Acid on Porcine Embryos Derived From in vitro Fertilization and Somatic Cell Nuclear Transfer. **Theriogenology**, v. 66, n. 9, p. 2104–2112, doi:10.1016/j.theriogenology.2006.06.007

KAIDI, S.; BERNARD, S.; LAMBERT, P.; MASSIP, P.; DESSY, F.; DONNAY, I. (2001). Effect of Conventional Controlled-Rate Freezing and Vitrification on Morphology and Metabolism of Bovine Blastocysts Produced in vitro. **Biol Reprod**, v. 65, p. 1127-1134.

KERE, M.; SIRIBOON, C.; LO, N.-W.; NGUYEN, N. T.; JU, J.-C. (2013) Ascorbic Acid Improves the Developmental Competence of Porcine Oocytes After Parthenogenetic Activation and Somatic Cell Nuclear Transplantation. **The Journal of Reproduction and Development,** v. 59, n. 1, p. 78–84. PMID: 23154385

KIMURA, H.; YAMADA, Y.; MORITA, Y.; IKEDA, H.; MATSUO, T. (1992) Dietary Ascorbic Acid Depresses Plasma and Low Density Lipoprotein Lipid Peroxidation in Genetically Scorbutic Rats. **Journal of Nutrition**, v. 122, n. 9, p. 1904–1909.

KORHONEN, K.; JULKUNEN, H.; KANANEN, K.; BREDBACKA, P.; TIIRIKKA, T.; RÄTY, M.; VARTIA, K.; KAIMIO, I.; KONTINEN, A.; HALMEKYTÖ, M.; VILKKI, J.; PEIPPO, J.; LINDEBERG, H. (2012) The Effect Of Ascorbic Acid During Biopsy and Cryopreservation on Viability of Bovine Embryos Produced in vivo. **Theriogenology**, v. 77, n. 1, p. 201–205. doi: 10.1016/j.theriogenology.2011. 07.034

LANE, M.; MAYBACH, J.; GARDNER, D. (2002) Addition of Ascorbate During Cryopreservation Stimulates Subsequent Embryo Development. **Human Reproduction**, v. 17, n. 10, p. 2686–2693. doi: 10.1093/humrep/17.10.2686

LEVINE, M.; MORITA, K. (1985) Ascorbic Acid in Endocrine Systems. **Vitamins & Hormones**, v. 42, p. 1–64. doi: 10.1016/S0083-6729(08)60060-6

LINDAHL, T.; WOOD, R. D. (1999) Quality Control by DNA Repair. **Science (New York, N.Y.)**, v. 286, n. 5446, p. 1897–1905. doi: 10.1126/science.286.5446.1897

LINSTER, C. L.; VAN SCHAFTINGEN, E. (2007) Vitamin C: Biosynthesis, Recycling and Degradation in Mammals. **FEBS Journal**, v. 274, n. 1, p. 1–22. doi: 10.1111/j.1742-4658.2006.05607.x

LONERGAN, P.; RIZOS, D.; KANKA, J.; NEMCOVA, L.; MBAYE, A. M.; KINGSTON, M.; WADE, M.; DUFFY, P.; BOLAND, M. P. (2003) Temporal Sensitivity of Bovine Embryos to Culture Environment After Fertilization and the Implications for Blastocyst Quality. **Reproduction**, v. 126, n. 3, p. 337–346. doi: 10.1530/reprod/126.3.337

LUCK, M. R.; JEYASEELAN, I.; SCHOLES, R. A. (1995) Ascorbic Acid and Fertility. **Biology of Reproduction**, v. 52, n. 2, p. 262–266. doi: 10.1095/biolreprod52.2.262

LUCK, M. R.; JUNGCLAS, B. (1987) Catecholamines and Ascorbic Acid as Stimulators of Bovine Ovarian Oxytocin Secretion. **Journal of Endocrinology,** v. 114, n. 3, p. 423–430. doi:10.1677/joe.0.1140423

LUCK, M. R.; ZHAO, Y. (1993) Identification and Measurement of Collagen in the Bovine Corpus Luteum and its Relationship with Ascorbic Acid and Tissue Development. **Journal of Reproduction and Fertility**, n. 99, p. 647–652. doi:035413/Z/92/Z

LUTWAK-MANN, C. (1954) Note on the Chemical Composition of Bovine Follicular Fluid. **A.R.C. Unit of Animal Reproduction**, School of Agriculture, University of Cambridge, v. 44, p. 477–480.

LUZ, V. B.; ARAUJO, V. R.; DUARTE, A. B. G.; CELESTINO, J. J. H.; SILVA, T. F. P.; MAGALHAES-PADILHA, D. M.; CHAVES, R. N.; BRITO, I. R.; ALMEIDA, A. P.; CAMPELLO, C. C.; FELTRIN, C.; BERTOLINI, M.; SANTOS, R. R.; FIGUEIREDO, J. R. (2012) Eight-Cell Parthenotes Originated From in vitro Grown Sheep Preantral Follicles. **Reproductive Sciences,** v. 19, n. 11, p. 1219–1225. doi:10.1177/1933719112446072.

MAGALHÃES, D. M.; DUARTE, A. B. G.; ARAÚJO, V. R.; BRITO, I. R.; SOARES, T. G.; LIMA, I. M. T.; LOPES, C. A. P.; CAMPELLO, C. C.; RODRIGUES, A. P. R.; FIGUEIREDO, J. R. (2011) in vitro Production of a Caprine Embryo From a Preantral Follicle Cultured in Media Supplemented

With Growth Hormone. **Theriogenology**, v. 75, n. 1, p. 182–188. doi: 10.1016/j.theriogenology.2010.08.004

MAHFOUZ, M. M.; KUMMEROW, F. A. (2004) Vitamin C or Vitamin B6 Supplementation Prevent The Oxidative Stress and Decrease of Prostacyclin Generation in Homocysteinemic Rats. **The International Journal of Biochemistry & Cell Biology**, v. 36, n. 10, p. 1919–1932. doi:10.1016/j.biocel.2004.01.028

MCLAUGHLIN, M.; TELFER, E.E. (2010) Oocyte development in bovine primordial follicles is promoted by activin and FSH within a two-step serum-free culture system. **Society for Reproduction and Fertility**, v. 139, p. 971-978.

MELO, M. A. P.; OSKAM, I. C.; CELESTINO, J. J. H.; CARVALHO, A. A.; CASTRO, S. V.; FIGUEIREDO, J. R.; RODRIGUES, A. P. R.; SANTOS, R. R. (2011) Adding Ascorbic Acid to Vitrification and IVC Medium Influences Preantral Follicle Morphology, but Not Viability. **Reproduction in Domestic Animals**, v. 46, n. 4, p. 742–745. doi: 10.1111/j.1439-0531.2010.01716.x

MURRAY, A. A.; MOLINEK, M. D.; BAKER, S. J.; KOJIMA, F. N.; SMITH, M. F.; HILLIER, S. G.; SPEARS, N. (2001) Role of Ascorbic Acid in Promoting Follicle Integrity And Survival in Intact Mouse Ovarian Follicles in vitro. **Reproduction**, v. 121, n. 1, p. 89–96. doi:10.1530/reprod/121.1.89

MYLLYLÄ, R.; KUUTTI-SAVOLAINEN, E.-R.; KIVIRIKKO, K. I. (1978) The Role of Ascorbate in the Prolyl Hydroxylase Reaction. **Biochemical and Biophysical Research Communications**, v. 83, n. 2, p. 441–448. doi:10.1016/0006-291X(78)91010-0

NEDAMBALE, T. L.; DU, F.; YANG, X.; TIAN, X. C. (2006) Higher Survival Rate of Vitrified and Thawed in vitro Produced Bovine Blastocysts Following Culture in Defined Medium Supplemented with B-Mercaptoethanol. **Animal Reproduction Science**, v. 93, n. 1–2, p. 61–75. doi:10.1016/j.anireprosci.2005.06.027

O'BRIEN, M. J. A.; PENDOLA, J. K.; EPPIG, J. J. (2003) Revised Protocol for in vitro Development of Mouse Oocytes from Primordial Follicles Dramatically Improves Their Developmental Competence. **Biology of Reproduction**, v. 68, n. 5, p. 1682–1686. doi 10.1095/biolreprod.102.013029

OGUNTIBEJU, O. O. (2008) The Biochemical, Physiological and Therapeutic Roles of Ascorbic Acid. **African Journal of Biotechnology**, v. 7(25), p. 4700-4705.

PADAYATTY, S. J.; KATZ, A.; WANG, Y.; ECK, P.; KWON, O.; LEE, J.-H.; CHEN, S.; CORPE, C.; DUTTA, A.; DUTTA, S. K.; LEVINE, M. (2003) Vitamin C as an Antioxidant: Evaluation of its Role in Disease Prevention. **Journal of the American College of Nutrition**, v. 22, n. 1 p. 18–35, doi:10.1080/07315724.2003.10719272

PADH, H. (1991) Vitamin C: Newer Insights into Its Biochemical Functions. **Nutrition Reviewsv.** 49, p. 65–70, doi: 10.1111/j.1753-4887.1991.tb07407.x

PATAK, P.; WILLENBERG, H. S.; BORNSTEIN, S. R. (2004) Vitamin C is an Important Cofactor for Both Adrenal Cortex and Adrenal Medulla. **Endocrine research,** v. 30, n. 4, p. 871–875, doi:10.1081/ERC-200044126

PINTAURO, S. J.; BERGAN, J. G. (1982) Effects of Ascorbic Acid on in vitro Steroidogenesis in Guinea Pigs. **The Journal of nutrition**, v. 112, n. 3, p. 584–591, PMID:7062153

RICHELLE, M.; SABATIER, M.; STEILING, H.; WILLIAMSON, G. (2006) Skin Bioavailability of Dietary Vitamin E, Carotenoids, Polyphenols, Vitamin C, Zinc and Selenium. **The British journal of nutrition,** v. 96, n. 2, p. 227–238. doi:10.1079/BJN20061817

RIZOS, D.; GUTIÉRREZ-ADÁN, A.; PÉREZ-GARNELO, S.; DE LA FUENTE, J.; BOLAND, M. P.; LONERGAN, P. (2003) Bovine Embryo Culture in the Presence or Absence of Serum: Implications for Blastocyst Development,

Cryotolerance, and Messenger RNA Expression. **Biology of reproduction,** v. 68, n. (1) p. 236-43.

ROOMI, M. W.; SHANKER, N.; NIEDZWIECKI, A.; RATH, M. (2015) Vitamin C in Health : Scientific Focus on its Anti-Cancer Eficacy. **Journal of Cellular Medicine and Natural Health,** p. 1–16.

ROSOL, T. J.; JOHN T. YARRINGTON, JOHN LATENDRESSE, A. C. C. C. (2001) Adrenal Gland : Structure , Function , and M echanisms of Toxicity. **Toxicologic Pathology,** v. 29, n. 1, p. 41–48.

ROSSETTO, R.; LIMA-VERDE, I. B.; MATOS, M. H. T.; SARAIVA, M. V. A.; MARTINS, F. S.; FAUSTINO, L. R.; ARAÚJO, V. R.; SILVA, C. M. G.; NAME, K. P. O.; SN, S. N. B.; CAMPELLO, C. C.; FIGUEIREDO, J. R.; BLUME, H. (2009) Interaction Between Ascorbic Acid and Follicle-Stimulating Hormone Maintains Follicular Viability After Long-Term in vitro Culture of Caprine Preantral Follicles. **Domestic Animal Endocrinology**, v. 37, n. 2, p. 112–123. doi:10.1016/j.domaniend.2009.04.003

ROTTA A. M. (2003) Utilização do Ácido Ascórbico (Vitamina C) pelos Peixes**.** **Embrapa Pantanal,** v. 1, p. 54. doi:1517-1973;49

RUIZ, A.; HANSEN, P.; BLOCK, J. (2015) 53 Effect of Addition of L-Carnitine and Conjugated Linoleic Acid During Bovine Embryo Culture on Cryosurvival, Lipid Content,. **Reproduction, Fertility and Development**, 27, p. 119–120. doi:10.1071/RDv27n1Ab53

SARAIVA, M. V. A.; ROSSETTO, R.; BRITO, I. R.; CELESTINO, J. J. H.; SILVA, C. M. G.; FAUSTINO, L. R.; ALMEIDA, A. P.; BRUNO, J. B.; MAGALHÃES, D. M.; MATOS, M. H. T.; CAMPELLO, C. C.; FIGUEIREDO, J. R. (2010) Dynamic Medium Produces Caprine Embryo from Preantral Follicles Grown in vitro. **Reproductive Sciences** (Thousand Oaks, Calif.), v. 17, n. 12, p. 1135–43. doi:10.1177/1933719110379269

SARSOUR, E. H.; KUMAR, M. G.; CHAUDHURI, L.; KALEN, A. L.; GOSWAMI, P. C. (2009) Redox Control of the Cell Cycle in Health and Disease. **Antioxidants & Redox Signaling**, v. 11, n. 12, p. 2985–3011. doi:10.1089/ars.2009.2513

SEIDEL, G. J. (2006) Modifying Oocytes and Embryos to Improve Their Cryopreservation. **Theriogenology**, v. 65, p. 228-235.

SERAFIM, M.K.B.; SILVA, G.M.; DUARTE, A.B.G.; ARAÚJO, V.R.; SILVA, T.F.P.; LIMA, A.K.F.; CHAVES, R.N.; CAMPELLO, C.C.; SILVA, L.D.M.; FIGUEIREDO, J.R. (2013) High Insulin Concentrations Promote the in vitro Growth and Viability of Canine Preantral Follicles. **Reproduction Fertility and Development,** v. 25, n. (6) p. 927-934 doi: 10.1071/RD12074.

SILVA, G. M.; ARAÚJO, V. R.; DUARTE, A. B. G.; CHAVES, R. N.; SILVA, C. M. G.; LOBO, C. H.; ALMEIDA, A. P.; MATOS, M. H. T.; TAVARES, L. M. T.; CAMPELO, C. C.; FIGUEIREDO, J. R. (2011) Ascorbic Acid Improves the Survival and in vitro Growth of Isolated Caprine Preantral Follicles. **Animal Reproduction**, v.8, n.1/2, p.14-24.

TAO, Y.; ZHOU, B.; XIA, G.; WANG, F.; WU, Z.; FU, M. (2004) Exposure to L-Ascorbic Acid or α-Tocopherol Facilitates the Development of Porcine Denuded Oocytes from Metaphase I to Metaphase II and Prevents Cumulus Cells from Fragmentation. **Reproduction in Domestic Animals,** v. 39, n. 1, p. 52–57. doi:10.1046/j.1439-0531.2003.00478.x

TARÍN, J. J.; TROUNSON, A. O. (1993) Effects of Stimulation or Inhibition of Lipid Peroxidation on Freezing-Thawing of Mouse Embryos. **Biology of reproduction,** v. 49, n. 6, p. 1362–1368. PMID: 8286618

TATEMOTO, H.; OOTAKI, K.; SHIGETA, K.; MUTO, N. (2001) Enhancement of Developmental Competence After in vitro Fertilization of Porcine Oocytes by Treatment with Ascorbic Acid 2-O-Alpha-Glucoside During in vitro Maturation.

Biology of Reproduction, v. 65, n. 6, p. 1800–1806, doi:10.1095/biolreprod65.6.1800

TELFER, E.E.; MCLAUGHLIN, M.; DING, C.; THONG, K.J. (2008) A Two-Step Serum-Free Culture System Supports Development of Human Oocytes from Primordial Follicles in the Presence of Activin. **Human Reproduction**, v. 23, p. 1151-1158.

THOMAS, F. H.; LEASK, R.; SRŠEN, V.; RILEY, S. C.; SPEARS, N.; TELFER, E. E. (2001) Effect of Ascorbic Acid on Health and Morphology of Bovine Preantral Follicles During Long-Term Culture. **Reproduction**, v. 122, n. 3, p. 487–495. doi:10.1530/reprod/122.3.487

TILLY, J. L.; TILLY, K. I. (1995) Inhibitors of Oxidative Stress Mimic the Ability of Follicle-Stimulating Hormone to Suppress Apoptosis in Cultured Rat Ovarian Follicles. **Endocrinology,** v. 136, n. 1, p. 242–252. doi:10.1210/endo.136.1.7828537

TOLBERT, B. M.; DOWNING, M.; CARLSON, R. W.; KNIGHT, M. K.; BAKER, E. M. (1975) Chemistry and Metabolism of Ascorbic Acid and Ascorbate Sulfate. **Annals of the New York Academy of Sciences**, v. 258, p. 48–69.

VAN WEZEL, I. L.; RODGERS, R. J. (1996) Morphological Characterization of Bovine Primordial Follicles and their Environment in vivo. **Biology of Reproduction**, v. 55, n. 5, p. 1003–1011. doi:10.1095/biolreprod55.5.1003

VENEREO GUTIÉRREZ, J. R. (2002) Daño Oxidativo, Radicales Libres y Antioxidantes. **Revista Cubana de Medicina Militar**, v. 31, n. 2, p. 126–133.

WANG, X.; PH, D.; FALCONE, T.; ATTARAN, M. (2002) Vitamin C and Vitamin E Supplementation Reduce Oxidative Stress – Induced Embryo Toxicity and Improve the Blastocyst. **Fertility and Sterility**, v. 78, n. 6, p.1272-1277. doi:S0015-0282(02)04236-X

WILSON, J. X. (2005) Regulation of Vitamin C Transport. **Annual Review of Nutrition**, v. 25, p. 105–25. doi:10.1146/annurev.nutr.25.050304.092647

WU, J.; EMERY, B.R.; CARREL, D.T. (2001) in vitro Growth, Maturation, Fertilization and Embryonic Development of Oocytes. **Biology of Reproduction**, v. 64(1) p.375-381.

ZOLINI, A. M.; CARRASCAL-TRIANA E. L.; RUIZ, A.; PENITENTE-FILHO J.M.; HANSEN, P.; TORRES C. A. A; BLOCK, J. (2015) 163 Effects of Serum and L - Carnitine on Development and Cryotolerance of Bovine Embryos Produced in vitro. **Reproduction, Fertility and Development**, v. 28(2), p. 211–212.

CHAPTER 2

Ascorbic acid improves the survival of bovine cryopreserved in vitro produced embryos

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Abstract

Antioxidants may be beneficial additives to cryopreservation media due to their ability to reduce oxidative stress caused by reactive oxygen species. The aim of this study was to determine if ascorbic acid addition to freezing medium improves the survival of IVP bovine embryos, through re-expansion and hatching rates, intracellular reactive oxygen species levels, total blastocyst cells number and DNA fragmentation after cryopreservation. Cumulus oocyte complexes were obtained from ovaries of slaughterhouse cows. Oocytes were in vitro matured, fertilized and cultured to day 7. Blastocyst and expanded blastocysts-stage embryos were randomly assigned to be subjected to slow freezing. For experiment 1, embryos were frozen in Hepes-TALP with 1.5 M Ethylene Glycol, 0.1 M Sucrose and different ascorbic acid concentrations (0.0; 0.1; 0.3 and 0.5 mM). Embryos were thawed and then cultured for 72 h. Embryos treated with 0.1 mM ascorbic acid showed higher re-expansion at 24, 48 and 72 h and hatching at 72 h compared to control embryos ($P<0.05$), thus concentration was considered as the optimal for the sequential experiments. For experiment 2 and 3, embryos were cryopreserved in freezing medium containing or not 0.1 mM ascorbic acid, then were thawed and cultured for 24 h. Intracellular reactive oxygen species levels were reduced ($P<0.001$) by ascorbic acid treatment (30.3 ± 2.4) compared with control (49.3 ± 1.9). There was no effect on the blastocyst total cells number blastocyst. However, 0.1 mM ascorbic acid reduced ($P<0.001$) the percentage of apoptotic cells and DNA fragmentation compared with the control group. In conclusion, addition of ascorbic acid (0.1 mM) in slow-freezing medium improves the cryosurvival of in vitro produced bovine embryos, reduces intracellular reactive oxygen species levels and DNA fragmentation.

Key words: Antioxidant; Blastocyst; Cryopreservation; in vitro produced.

Introduction

In vitro production (IVP) of embryos has being used successfully to meet the needing for multiplication of bovine animals of superior genetics. This reproductive technique is able to accelerate the genetic gain by shorten the generation interval since at the estrous cycle only one oocyte develops towards ovulation (Fortune, 1994). With the use of IVP, it is expected to get many oocytes, which will be degenerated normally in vivo that could be converted to embryos.

It is possible to preserve embryos from animals of high genetic value by cryopreservation method. However, events during this procedure as exposure to cryoprotectants, temperature reduction and subsequent thawing of embryos can lead to cell damage by means the increase in reactive oxygen species (ROS; Tatone et al., 2010). ROS are free radicals produced as by-products of oxidation–reduction reactions. When the physiological balance between ROS production and antioxidant defenses is lost, oxidative stress will result in molecular damage to DNA, proteins and lipids within the cell (Gupta et al., 2009). The oxidative injury may induce release of cytochrome c and other apoptogenic factors from cell mitochondria which eventually activate programmed cell death, apoptosis (Orrenius et al., 2007). Therefore, in order to minimize such damage, improvement of the solutions used in cryopreservation is needed. There are strong indications that injuries caused by lipid peroxidation can be reduced by the use of antioxidants (Lane et al., 2002).

Ascorbic acid (AA) is an antioxidant and it is involved in processes of hormone production, gonadal tissue remodeling and apoptosis by its ability to protect cells from free radicals (Luck et al., 1995). It has also been demonstrated the efficient action of AA in embryos production. AA improved cytoplasmic maturation of bovine oocytes (Córdova et al., 2010); the development of embryos in mouse (Wang et al., 2002; Lane et al., 2002), porcine (Jeong et al., 2006; Hossein et al., 2007; Kere et al., 2013, Castillo-Martín et al., 2014a) and bovine (Ferro et al., 2009; Córdova et al., 2010) and also results in a significantly increased embryo survival to the freezing-thawing stress (Tarín and Trounson, 1993). Despite the advantages offered by addition of AA in embryo production protocols, the ideal concentration of AA in cryopreservation solutions of bovine embryos has not been defined yet. Lane et al. (2002) indicate that the addition of 0.1 mM of AA to the cryopreservation solutions significantly enhanced the ability of mouse embryos both at the cleavage and blastocyst stages to survive cryopreservation. It reduced the levels of hydrogen

peroxide and increased inner cell mass development in blastocyst stage embryos. Therefore, it is plausible that use of the same or different AA concentrations in the bovine specie may help in maintaining cryopreserved IVP embryo viability.

The aim of this study was to determine if AA addition to freezing medium improves the survival of IVP bovine embryos, through examination of embryo quality parameters, such as re-expansion and hatching rates, intracellular ROS levels, total blastocyst cells number and DNA fragmentation after cryopreservation.

Materials and Methods

All experimental procedures were approved by the Ethic Commission in use of Production Animals of Universidade Federal de Viçosa, Brazil (process number 41/2014).

Experimental Design

Experiment 1: Effect of AA antioxidant on re-expansion and hatching rate of cryopreserved IVP bovine embryos.

Bovine embryos were produced by in vitro fertilization of oocytes obtained from abattoir-collected ovaries. Immature cumulus oocyte complexes (COC's) were obtained by slicing follicles. Presumptive zygotes, 18-20 h post-insemination were placed in groups of 25-30 in 50 µL drops of synthetic oviduct fluid-bovine embryo 1 (SOF-BE1 – Fields et al., 2011). At day 7 after insemination, the embryos at the blastocyst and expanded blastocyst stage evaluated as Grade 1 and 2 (according to the IETS manual, Stringfellow and Seidel, 1998) were harvested from culture. Embryos were subjected to controlled-rate freezing following equilibration for 10 min in freezing medium (Hepes-Tyrode's Albumin Lactate Pyruvate (Hepes-TALP) with 1.5 M ethylene glycol, 0.1 M Sucrose) and different AA concentrations 0.0 mM, 0.1 mM, 0.3 mM and 0.5 mM (in Annex). After exposure, the embryos were loaded in groups of six to 10 per straw of 0.25 mL. The experiment was replicated 10 times; harvested embryos (578) were used to determine re-expansion and hatching rates of bovine embryos during 24, 48 and 72 h post-thaw. There were between 141 and 148 embryos for each treatment.

Experiment 2: Effects of AA antioxidant on intracellular ROS levels of cryopreserved IVP bovine embryos.

Bovine embryos were produced in vitro using abattoir-derived ovaries. At Day 7 after insemination, Grade 1 and 2 blastocysts and expanded blastocysts stage embryos were harvested and frozen conventionally in freezing medium containing or not 0.1 mM of AA (based on results of survival post-thaw from first experiment). Embryos were thawed and then cultured for 24 h. Harvested embryos (63) in total (30 for the control and 33 embryos for AA) were analysed by CellROX Green reagent to determine the oxidative stress caused by intracellular ROS levels. The experiment was replicated 6 times.

Experiment 3: Effects of AA antioxidant on total cells number of blastocysts and DNA fragmentation of cryopreserved IVP bovine embryos.

Bovine embryos were produced in vitro using abattoir-derived ovaries. At Day 7 after insemination, Grade 1 and 2 blastocysts and expanded blastocysts stage embryos were harvested and subjected to slow freezing in medium containing or not 0.1 mM of AA (based on results of survival post-thaw from first experiment). Embryos were thawed and then cultured for 24 h. Harveste

d embryos (166; 79 for the control and 87 embryos for AA) were analyzed by the terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) assay to detect the total cell number of the blastocysts and DNA fragmentation. The experiment was replicated 6 times.

In vitro Embryo Production

Bovine embryos were produced by in vitro fertilization of oocytes obtained from abattoir-collected ovaries. All procedures for embryo production followed techniques previously described by Fields et al. (2011). COC's were obtained by slicing follicles from 2 to 10 mm in diameter on the surface of ovaries into a beaker containing oocyte washing medium (Mofa, Verona, WI, USA). Groups of 10 COC's were matured in 50 μL droplets of oocyte maturation medium [tissue culture medium-199 with Earle’s salts (Invitrogen, Carlsbad, CA, USA), 10 % (v/v) bovine steer serum, 2 μg/mL estradiol 17-β, 20 μg/mL bovine follicle stimulating hormone

(Bioniche Life Sciences, Ontario, Canada), 22 µg/mL sodium pyruvate, 50 µg/mL gentamicin sulfate and 1 mM GlutaMAX™ (Life Technologies, Carlsbad, CA, USA)] covered with mineral oil for 20 hours at 38.5 °C and in a humidified atmosphere of 5 % (v/v) CO_2. Following maturation, COC's were washed once in Hepes-TALP (Caisson Laboratories, Inc., Logan, UT, USA; Parrish et al., 1986) and then transferred in groups of 200-300 to 35 mm Petri dishes containing 1.7 mL of in vitro fertilization-TALP (Caisson Laboratories, Inc., Logan, UT, USA; Parrish et al., 1986) and 120 µL of a mixture of 0.5 mM penicillamine, 0.25 mM hypotaurine and 25 µM epinephrine in 0.9% (w/v) NaCl and fertilized with 1 x 10^6 /mL spermatozoa purified from a pool of frozen-thawed conventional semen from three bulls using Isolate® separation gradient (Irvine Scientific, Santa Ana, CA, USA) for 18 - 20 h at 38.5 °C in a humidified atmosphere of 5% (v/v) CO_2. Mature oocytes were denuded after fertilization by vortexing in 600 µL Hepes-TALP containing 10,000 U/mL hyaluronidase and placed in groups of 25-30 in 50 µL microdrops of SOF-BE1 (Fields et al., 2011) covered with mineral oil at 38.5 °C in a humidified atmosphere of 5 % O_2 and 5 % CO_2 and 90% N_2. At Day 7 after insemination, embryos were harvested and subjected to slow freezing.

Slow-freezing procedure

Blastocyst and expanded blastocysts grade 1 and 2 according to the guidelines of the International Embryo Transfer Society (IETS; Stringfellow and Seidel, 1998) were harvested from culture on Day 7 (Day 0 = day of in vitro fertilization), washed three times in Hepes-TALP at room temperature and then transferred directly into the freezing medium (Hepes-TALP plus 1.5 M ethylene glycol (Sigma Aldrich, St. Louis, MO, USA) and 0.1 M Sucrose (Sigma Aldrich, St. Louis, MO, USA)] with AA treatments (A7631 Sigma Aldrich, St. Louis, MO, USA). Embryos were loaded in groups of six to 10 per straw of 0.25 mL with three columns of freezing medium separated by 5 mm air columns and embryos were loaded into the middle column of straws and allowed to equilibrate for 10 min.

The embryos into straws were then placed into the chamber of a programmable freezing machine (CryoLogic Freeze Control® CL-5500, Victoria, Australia) set at -6.0 °C and after 2 min, straws were seeded by touching the top column of medium with a cotton swab dipped in liquid nitrogen. The straws were

maintained at this temperature for 10 min, and then cooled to -32 °C at 0.5 °C per min and then held for 10 min prior to being plunged into liquid nitrogen (-196 °C).

The slow freezing method was chosen over vitrification because frozen embryos through this technique can be thawed without the need of laboratory conditions and specialized technicians. Therefore, more likely to be used in farm conditions.

Post Thaw Embryo Survival

At the time of thawing, embryos were removed from liquid nitrogen and held in the air at room temperature for 5 seconds, followed by immersion into a water-bath at 30 °C for 20 seconds. Embryos were washed three times in Hepes-TALP at room temperature and cultured for 72 h in groups of 8-14 in 25 µL microdrops of SOF-BE1 supplemented with 10% fetal bovine serum overlaid with mineral oil at 38.5 °C in a humidified atmosphere of 5 % (v/v) CO_2, 5 % (v/v) O_2 and 90% N_2. Re-expansion and hatching rates (including both hatching and hatched embryos) were recorded at 24, 48 and 72 h post-thaw.

Oxidative stress analysis by CellROX Green

CellROX® Green Reagent (Molecular Probes, Eugene, OR, USA) is a DNA dye, the fluorescent probe penetrates the cell and, when oxidized by intracellular free radicals, binds to DNA, emitting an intense green fluorescence. For this assay, embryos were washed twice in PBS and incubated with 5 µM (final concentration) of cellROX® for 30 minutes in the dark at 38.5 °C and 5% CO_2 in humidified air. Further, they were washed twice in PBS and were placed in 3.7% formaldehyde for 15 minutes at room temperature. Following fixation, embryos were washed again in PBS and mounted on glass slides surrounded with 5µL of ProLong® Gold antifage around the embryo and then carefully covered with a cover slip. Stained embryos were immediately evaluated in inverted microscope and analyzed by epifluorescence program Image J.

TUNEL and Hoescht 33342 Labeling

The terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) assay was used to detect DNA fragmentation that is associated with the late stages of apoptosis. The enzyme terminal deoxynucleotidyl transferase was used to catalyze the transfer of a fluorescein isothiocyanate-conjugated dUTP nucleotide to the free 3' hydroxyl group that is exposed after DNA cleavage. Embryos were removed from SOF-BE1 and washed two times in 50-µL drops of PBS-PVP by transferring the embryos from drop to drop. Embryos were fixed in a 50-µL drop of 4% (w/v) paraformaldehyde in PBS for 1 hour at room temperature, washed twice in PBS-PVP. Embryos were then permeabilized in 50 µL drop of 0.5% (v/v) Triton X-100, 0.1% (w/v) sodium citrate for 30 min at room temperature in a humidified box. After permeabilization, embryos were washed three times in 50 µL drops of PBS-PVP. Embryos cultured under standard conditions were used as positive and negative controls for the TUNEL procedure. Positive and negative controls were treated in 50 µL drops of RQ1-RNase-free DNase (50 U/mL; Promega, Madison, WI, USA) at 37 °C in the dark for 1 h. Positive controls and experimental embryos were washed in PBS-PVP and incubated with 25 µL of TUNEL reaction mixture (In Situ Cell Death Detection Kit, Fluorescein: Roche Diagnostics Corporation, Indianapolis, IN, USA) (containing fluorescein- conjugated dUTP and the enzyme terminal deoxynucleotidyl transferase) for 1 h at 37 °C in the dark. Negative controls were incubated in the absence of terminal deoxynucleotidyl transferase. Embryos were washed three times in PBS-PVP and incubated in a 25 µL drop of Hoechst 33342 (1 µg/mL) for 15 min at room temperature. Embryos were washed three times, mounted on a microscope slide using ProLong® Gold Anti-Fade mounting medium (Thermo Fisher Scientific Inc), and fluorescence was visualized using a Zeiss Axioplan microscope (Zeiss, Göttingen, Germany) with a 20x objective and the FITC, DAPI, and DIC filter sets. Digital images were acquired using the AxioVision software and a high-resolution black and white AxioCam MRm digital camera (Zeiss).

Statistical analysis

For data analysis, the Statistical Analysis System (SAS, version 9.3; SAS Institute Inc., Cary, NC, USA) was used. Re-expansion and hatching rates were evaluated by logistic regression considering treatment and replication effects. For

discussion of results only the effect of treatment was considered. Intracellular ROS levels, total cells number of blastocyst and DNA fragmentation obtained were subjected to analysis of variance using the generalized linear model procedure (PROC GLM). Replicate was considered a random variable and treatment was considered a fixed variable in all models. Differences in treatment means were determined using a t-test in the PDIFF option. Results are presented as least squares means ± SEM. The level of significance used in all analyses was 0.05.

Results

Experiment 1. Effect of AA antioxidant on re-expansion and hatching rate of cryopreserved IVP bovine embryos.

Re-expansion and hatching rates for IVP embryos cryopreserved in freezing medium containing or not AA are shown in Table 1. Embryos in the presence of 0.1 and 0.3 mM AA exhibited higher ($P<0.05$) re-expansion rates at 24 and 72 h post thawing, however no differences ($P>0.05$) were observed between 0.3 and 0.5 mM AA at same time of culture. There were no differences ($P<0.05$) among AA treatments at 48 h of re-expansion and 48 and 72 h of hatching. As 0.1 mM AA was the only treatment that showed better re-expansion at 24, 48 and 72 h and hatching at 72 h compared with those embryos cryopreserved in absence of AA, it was considered as the better AA concentration for the sequential experiments.

Experiment 2: Effects of AA antioxidant on intracellular ROS levels of cryopreserved IVP bovine embryos.

Embryos cryopreserved in the absence of AA (control) resulted in higher intracellular ROS levels ($P<0.001$) compared to the 0.1 mM AA treatment at 24 h post-thaw (Figure 1).

Experiment 3: Effects of AA antioxidant on total cells number of blastocyst and DNA fragmentation of cryopreserved IVP bovine embryos.

The TUNEL assay revealed the percentage of DNA fragmentation, presence of apoptotic cells and total cells number of embryos cryopreserved in slow freezing

containing or not AA (Table 2). There was no effect on the total cells number of blastocyst at 24 hours of culture after thawing. However, 0.1 mM AA supplementation during the cryopreservation reduced ($P<0.001$) the percentage of apoptotic cells and DNA fragmentation compared with the control group.

Discussion

In the present study, the antioxidant AA was tested for the first time in slow freezing solutions of IVP embryos in bovine specie. The concentrations of AA (0.1 and 0.5 mM) used herein were chosen based on previous studies that showed satisfactory results after slow-freezing of mouse embryos at the 2-cell and blastocyst stages (Lane et al., 2002). In addition to these concentrations, it was also included 0.3 mM intermediate concentration of AA to provide a dose-concentration curve, which had not been elucidated yet. Cryopreserved bovine blastocyst and expanded blastocysts in the presence of low AA concentration, 0.1 mM in slow freezing solutions, significantly increased re-expansion and hatching rates, reduced ROS production and DNA fragmentation compared with the control treatment.

Cryopreservation has emerged as an acceptable method for preservation of reproductive cells. However, the process of freezing and thawing affects cell function by increasing the generation of ROS, thereby creating oxidative stress and augmenting oxidative metabolism (Agarwal et al., 2014). Oxidative stress can cause damage to cellular macromolecules, including nucleic acids, phospholipids, and proteins (Gupta et al., 2009). Oxidative damage to DNA causes modification of the purine and pyrimidine bases, the deoxyribose backbone; ROS induces lipid peroxidation and oxidation of proteins leads to loss of their normal functions and to enhanced susceptibility to proteolytic degradation (Orrenius et al., 2007). Despite the occurrence of the cellular events mentioned above, cells have natural defense mechanisms against oxidative stress comprising the action of antioxidants, which act inhibiting ROS production or capturing and inactivating them (Veal et al., 2007). Antioxidant supplementation has been used during different stages of in vitro embryo production to minimize the effects of oxidative stress, as the inclusion in the culture medium of melatonin (Dall'Acqua et al., 2015), α-tocopherol (Hossein et al., 2007; Saikhun et al., 2008), cysteamine (de Matos et al., 2002), catalase (Rocha-Frigoni, et al., 2013), b-mercaptoethanol (Kitagawa et al., 2004) and ascorbic acid (Wang et al.,

2002; Jeong et al., 2006; Hossein et al., 2007; Ferro et al., 2009; Kere et al., 2013). Nevertheless, there are few studies found in the literature about modification of freezing medium with antioxidants. In this study the ethylene glycol (1.5 M) was used as intracellular agent cryoprotectant which penetrate inside the cell preventing the formation of ice crystals, the sucrose (0.1 M) as extracellular agent that do not penetrate the cell membrane and act to improve the osmotic imbalance that occurs during freezing and AA (0.1 mM) as antioxidant to prevent oxidative stress and DNA fragmentation that ultimately improves survival of IVP bovine embryos. This result was similar to that found by Lane et al. (2002) in mouse embryos. For freezing, blastocysts were placed initially in MOPS–G2 medium (3-(N-morpholino) propanesulphonic acid with 0.1 and 0.5 mM of AA) plus 5% glycerol and 0.1 M sucrose for 10 min followed by 7 min incubation in MOPS–G2 with 10% glycerol and 0.2 M sucrose. Blastocysts were then loaded into a 0.25 mL straw and placed into the freezing machine at –6 °C to –32 °C, before being plunged into liquid nitrogen. They indicate that the addition of 0.1 mM AA to the cryopreservation solutions significantly enhances the survival of mouse embryos, reduced the levels of hydrogen peroxide and also stimulated the development the inner cell mass of embryos. Korhonen et al. (2012) based on the encouraging results of Lane et al. (2002) selected 0.1 mM AA exposure during biopsy and freezing medium of bovine embryos produced in vivo. After biopsy, embryos were frozen individually in straws either with 1.5 M ethylene glycol (control group) or ethylene glycol supplemented with 0.1 mM AA using a bath freezer at –7 °C to –35 °C. Then, female embryos were transferred to recipients. The results showed an appreciable increase in calving rate (22% for control embryos and 31% for AA embryos). Therefore, the addition of AA into the freezing medium is beneficial for survival and implantation of embryos regardless of the freezing method used.

It is known that AA is involved in many cellular reactions, as protection against lipid peroxidation, the repair of oxidized amino acids for protein integrity maintenance (Barja et al., 1994), inhibition of apoptosis in bovine granulosa cells and murine cumulus-oocyte complexes (Tilly & Tilly, 1995, Eppig et al., 2000, Murray et al, 2001), increase gene expression of antioxidant enzymes, such as SOD1 and GPX1, which reduce intracellular levels of ROS in IVP porcine blastocysts (Castillo-Martín et al., 2014b) and reduces levels of heat shock protein 70 (HSPA1A) in vitrified embryos (Castillo-Martín et al., 2014c). Additionally in the present study it

was demonstrated that AA is able to act on intracellular ROS levels, promoting embryo survival after cryopreservation by the DNA fragmentation protection that is associated with the late stages of apoptosis. The efficient action of AA could be explained because it is a reducing agent. AA may lose the hydrogen ions attached to one of its two ionizable groups located at carbons 2′ and 3′, generating ascorbate monoanion or dianion, dehydroascorbic acid (Tolbert et al., 1975; Du et al., 2012; Figueroa-Méndez and Rivas-Arancibia, 2015). Furthermore, this conversion is reversible and catalyzed by dehydroascorbate reductase enzyme or through spontaneous reaction with glutathione (Englard and Seifter 1986; Bánhegyi et al., 1997; Linster and Van Schaftingen, 2007). In vitro studies have found that dehydroascorbic acid enters the cell via high-capacity glucose transporters (GLUTs – hexose transporters) and is reduced intracellularly to AA. Ascorbic acid is also directly taken up by the cells through specific, low-capacity sodium ascorbate transporters (SVCTs – sodium-ascorbate cotransporter; Agus et al., 1997; Wilson, 2005; Roomi et al., 2015) and in this way confers mitochondrial protection against oxidative injury (Sagun et al., 2005).

Conclusion

The present study provides the first evidence that the use of 0.1 mM of AA in slow-freezing medium improves the survival of bovine cryopreserved in vitro produced embryos; AA antioxidant supplements can reduce intracellular ROS production and DNA fragmentation.

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References

AGARWAL, A.; DURAIRAJANAYAGAM, D.; DU PLESSIS, S. S., (2014) Utility of Antioxidants During Assisted Reproductive Techniques: An Evidence Based Review. **Reproductive Biology and Endocrinology**. 12, 112. doi:10.1186/1477-7827-12-112

AGUS, D. B.; GAMBHIR, S. S.; PARDRIDGE, W. M.; SPIELHOLZ, C.; BASELGA, J.; VERA, J. C.; GOLDE, D. W. (1997) Vitamin C Crosses the Blood-Brain Barrier in the Oxidized Form Through the Glucose Transporters. **The Journal of clinical investigation**, v. 100, n. 11, p. 2842–2848. doi: 10.1172/JCI119832

BÁNHEGYI, G., BRAUN, L., CSALA, M., PUSKÁS, F., MANDL, J. (1997) Ascorbate Metabolism and its Regulation in Animals. **Free Radical Biology and Medicine**. v. 23, n. 5, p. 793-803. doi: 10.1016/S0891-5849(97)00062-2

BARJA, G.; LÓPEZ-TORRES, M.; PÉREZ-CAMPO, R.; ROJAS, C.; CADENAS, S.; PRAT, J.; PAMPLONA, R. (1994) Dietary Vitamin C Decreases Endogenous Protein Oxidative Damage, Malondialdehyde, and Lipid Peroxidation and Maintains Fatty Acid Unsaturation in the Guinea Pig Liver. **Biology and Medicine,** v. 17, n. 2, p. 105–115. doi: 0891-5849(94)E0026-F

CASTILLO-MARTÍN, M.; YESTE, M.; SOLER, A.; MORATÓ, R, S. B. (2014a) Comparative Effects of Adding β-Mercaptoethanol or L-Ascorbic Acid to Culture or Vitrification-Warming Media on IVF Porcine Embryos. **Reproduction, Fertility and Development**, v. 26, n. 6, p. 875–882. doi:10.1071/RD13116

CASTILLO-MARTÍN, M.; BONET, S.; MORATÓ, ROSER.; M.; YESTE. (2014b) Supplementing Culture and Vitrification-Warming Media with L-Ascorbic Acid Enhances Survival Rates and Redox Status Of IVP Porcine Blastocysts Via Induction Of GPX1 and SOD1 Expression. **Cryobiology,** v. 68, n. 3, p. 451–458. doi:10.1016/j.cryobiol.2014.03.001

CASTILLO-MARTÍN, M.; YESTE, M.; SOLER, A.; MORATÓ, ROSER; BONET, S. (2014c) Addition of L-Ascorbic Acid to Culture and Vitrification Media of IVF Porcine Blastocysts Improves Survival and Reduces HSPA1A Levels of Vitrified Embryos. **Reproduction, Fertility and Development**, v. 27, n. 7, p. 1115–1123. doi: 10.1071/RD14078

CÓRDOVA, B.; MORATÓ, R.; IZQUIERDO, D.; PARAMIO, T.; MOGAS, T. (2010) Effect of the Addition of Insulin-Transferrin-Selenium and/or L-Ascorbic Acid to the in vitro Maturation of Prepubertal Bovine Oocytes on Cytoplasmic Maturation and Embryo Development. **Theriogenology**, v. 74, n. 8, p. 1341–1348. doi:10.1016/j.theriogenology.2010.06.003

DALL'ACQUA P.C., LEÃO B.C.S, ROCHAFRIGONI N. A. S., AMBROGI M., MINGOTI G.Z. (2015) Effects of Melatonin on Reactive Oxygen Species Generation and Acquisition of Embryonic Developmental Competence of Bovine Oocytes Matured in vitro. **Reproduction, Fertility and Development.** V.28 p.231-231. doi:10.1071/RDv28n2Ab200

DE MATOS, D.G.; HERRERA, C.; CORTVRINDT, R.; SMITZ, J.; VAN SOOM, A.; NOGUEIRA, D.; PASQUALINI, R.S. (2002) Cysteamine Supplementation during in vitro Maturation and Embryo Culture: A Useful Tool for Increasing the Efficiency of Bovine in vitro Embryo Production. **Mol Reprod Dev.** v.62, p.203-209. doi: 10.1002/mrd.10087

DU, J.; CULLEN, J. J.; BUETTNER, G. R. (2012) Ascorbic Acid: Chemistry, Biology and the Treatment of Cancer. **Biochimica et Biophysica Acta**, v. 1826, n. 2, p. 443–457. doi:10.1016/j.bbcan.2012.06.003

ENGLARD, S.; SEIFTER, S. (1986) The Biochemical Functions of Ascorbic Acid. **Annual Review of Nutrition,** v. 6, p. 365–406. doi:10.1146/annurev.nutr.6.1.365

EPPIG, J. J.; HOSOE, M.; O'BRIEN, M. J.; PENDOLA, F. M.; REQUENA, A.; WATANABE, S. (2000) Conditions that Affect Acquisition of Developmental

Competence by Mouse Oocytes in vitro: FSH, Insulin, Glucose and Ascorbic Acid. **Molecular and Cellular Endocrinology**, v. 163, n. 1–2, p. 109–116. doi: 10.1016/S0303-7207(99)00247-6

FERRO, A. C.; SILVA, C.; CALEGARI, R. S. (2009) Produção de Embriões in vitro em Meio de Cultura com Ácido Ascórbico. **Veterinaria e Zootecnia**, v. 16, n. 1, p. 80–88.

FIELDS, S. D.; HANSEN P. J.; EALY A. D. (2011) Fibroblast Growth Factor Requirements for in vitro Development of Bovine Embryos. **Theriogenology**, v.75, p. 1466–1475. doi: 10.1016/j.theriogenology.2010.12.007

FIGUEROA-MÉNDEZ, R. and RIVAS-ARANCIBIA, S. (2015) Vitamin C in Health and Disease: Its Role in the Metabolism of Cells and Redox State in the Brain. **Frontiers in Physiology.** v. 6, p. 397. doi: 10.3389/fphys.2015.00397

FORTUNE J.E. (1994) Ovarian Follicular Growth and Development in Mammals. **Biology of reproduction.** v.50, p. 225-232. doi: 10.1095/biolreprod50.2.225

GUPTA, S.; MALHOTRA, N.; SHARMA, D.; CHANDRA, A.; ASHOK, A. (2009) Oxidative Stress and its Role in Female Infertility and Assisted Reproduction: Clinical Implications. **Royan Institue International Journal of Fertility and Sterility**, v. 2, n. 4, p. 147–164.

HOSSEIN, M. S.; HASHEM, M. A.; JEONG, Y. W.; LEE, M. S.; KIM, S.; KIM, J. H.; KOO, O. J.; PARK, S. M.; LEE, E. G.; PARK, S. W.; KANG, S. K.; LEE, B. C.; HWANG, W. S. (2007) Temporal Effects of A-Tocopherol and L-Ascorbic Acid on in vitro Fertilized Porcine Embryo Development. **Animal Reproduction Science**, v. 100, n. 1–2, p. 107–117. doi:10.1016/j.anireprosci.2006.06.013

JEONG, Y. W.; PARK, S. W.; HOSSEIN, M. S.; KIM, S.; KIM, J. H.; LEE, S. H.; KANG, S. K.; LEE, B. C.; HWANG, W. S. (2006) Antiapoptotic and Embryotrophic Effects of B-Tocopherol and L-Ascorbic Acid on Porcine

Embryos Derived From in vitro Fertilization and Somatic Cell Nuclear Transfer. **Theriogenology**, v. 66, n. 9, p. 2104–2112. doi:10.1016/j.theriogenology.2006.06.007

KERE, M.; SIRIBOON, C.; LO, N.-W.; NGUYEN, N. T.; JU, J.-C. (2013) Ascorbic Acid Improves the Developmental Competence of Porcine Oocytes After Parthenogenetic Activation and Somatic Cell Nuclear Transplantation. **The Journal of Reproduction and Development,** v. 59, n. 1, p. 78–84. PMID: 23154385

KITAGAWA, Y.; SUZUKI, K.; YONEDA, A.; WATANABE, T. (2004) Effects Of Oxygen Concentration and Antioxidants on the in vitro Developmental Ability, Production of Reactive Oxygen Species (ROS), and DNA Fragmentation in Porcine Embryos. **Theriogenology.** v.62, p. 1186–1197. doi: 10.1016/j.theriogenology.2004.01.011

KORHONEN, K.; JULKUNEN, H.; KANANEN, K.; BREDBACKA, P.; TIIRIKKA, T.; RÄTY, M.; VARTIA, K.; KAIMIO, I.; KONTINEN, A.; HALMEKYTÖ, M.; VILKKI, J.; PEIPPO, J.; LINDEBERG, H. (2012) The Effect Of Ascorbic Acid during Biopsy and Cryopreservation on Viability of Bovine Embryos Produced in vivo. **Theriogenology**, v. 77, n. 1, p. 201–205. doi: 10.1016/j.theriogenology.2011.07.034

LANE, M.; MAYBACH, J.; GARDNER, D. (2002) Addition of Ascorbate During Cryopreservation Stimulates Subsequent Embryo Development. **Human Reproduction**, v. 17, n. 10, p. 2686–2693. doi: 10.1093/humrep/17.10.2686

LINSTER, C. L.; VAN SCHAFTINGEN, E. (2007) Vitamin C: Biosynthesis, Recycling and Degradation in Mammals. **FEBS Journal**, v. 274, n. 1, p. 1–22. doi: 10.1111/j.1742-4658.2006.05607.x

LUCK, M. R.; JEYASEELAN, I.; SCHOLES, R. A. (1995) Ascorbic Acid and Fertility. **Biology of Reproduction**, v. 52, n. 2, p. 262–266. doi: 10.1095/biolreprod52.2.262

MALLOL A., SANTALÓ J., IBÁÑEZ E. (2015) Improved Development of Somatic Cell Cloned Mouse Embryos by Vitamin C and Latrunculin A. **PLoS ONE**, v.10, p. 1-21. doi: 10.1371/journal.pone.0120033.

MURRAY, A. A.; MOLINEK, M. D.; BAKER, S. J.; KOJIMA, F. N.; SMITH, M. F.; HILLIER, S. G.; SPEARS, N. (2001) Role of Ascorbic Acid in Promoting Follicle Integrity And Survival in Intact Mouse Ovarian Follicles in vitro. **Reproduction**, v. 121, n. 1, p. 89–96. doi:10.1530/reprod/121.1.89

NATARAJAN, R.; BHAWANI, S. M.; MUNUSWAMY, D. (2010) Effect Of L-Ascorbic Acid Supplementation at Different Gaseous Environments on in vitro Development of Preimplantation Sheep Embryos to the Blastocyst Stage. **Journal of Assisted Reproduction and Genetics,** v.27, p. 483–490. doi: 10.1007/s10815-010-9430-7

NASR-ESFAHANI, M. H.; AITKEN, J. R.; JOHNSON, M. H. (1990) Hydrogen-Peroxide Levels in Mouse Oocytes and Early Cleavage Stage Embryos Developed in vitro or in vivo. **Development,** v.109, p.501–507. Doi: 1990 109: 501-507

ORRENIUS, S.; GOGVADZE, V.; ANNU, B. Z. (2007) Mitochondrial Oxidative Stress: Implications for Cell Death. **Rev. Pharmacol. Toxicol**. v. 47, p. 143–83. doi: 10.1146/annurev.pharmtox.47.120505.105122

PADAYATTY, S. J.; KATZ, A.; WANG, Y.; ECK, P.; KWON, O.; LEE, J.-H.; CHEN, S.; CORPE, C.; DUTTA, A.; DUTTA, S. K.; LEVINE, M. (2003) Vitamin C as an Antioxidant: Evaluation of its Role in Disease Prevention. **Journal of the American College of Nutrition**, v. 22, n. 1 p. 18–35. doi:10.1080/07315724.2003.10719272

PARRISH, J. J.; SUSKO-PARRISH, J. L.; LEIBFRIED-RUTLEDGE, M. L. CRITSER, E. S.; EYESTONE, W. H.; FIRST N. L. (1986) Bovine in vitro Fertilization with Frozen-Thawed Semen. **Theriogenology**. v.25, p. 591–600. doi: 10.1016/0093-691X(86)90143-3

ROCHA-FRIGONI, N. A.; LEÃO, B. C.; NOGUEIRA, É.; ACCORSI, M.F.; MINGOTI, G. Z. (2013) Reduced Levels of Intracellular Reactive Oxygen Species and Apoptotic Status are not Correlated with Increases in Cryotolerance of Bovine Embryos Produced in vitro in the Presence of Antioxidants. **Reproduction, Fertility and Development.** v. 26, p. 797-805. doi: 10.1071/RD12354.

ROOMI, M. W.; SHANKER, N.; NIEDZWIECKI, A.; RATH, M. (2015) Vitamin C in Health : Scientific Focus on its Anti-Cancer Eficacy. **Journal of Cellular Medicine and Natural Health,** p. 1–16.

SAIKHUN, K.; FAISAIKARM, T.; MING, Z.; LU, K.H.; KITIYANANT, Y. (2008) α-Tocopherol and L-Ascorbic Acid Increase the in vitro Development of IVM/IVF Swamp Buffalo (Bubalus Bubalis) Embryos. **Animal**, v.2, p.1486-1490. doi: 10.1017/S1751731108002541

SAGUN, K.C.; CARCAMO, J.M.; GOLDE, D.W. (2005) Vitamin C enters Mitochondria via Facilitative Glucose Transporter 1 (Glut1) and Confers Mitochondrial Protection Against Oxidative Injury. **The FASEB J,** v.19 p.1657-1667. doi: 10.1096/fj.05-4107com

STRINGFELLOW D. A.; SEIDEL S. M. (1998) ‘Manual of the International Embryo Transfer Society: A Procedural Guide and General Information for the Use of Embryo Transfer Technology Emphasizing Sanitary Procedures’. **International Embryo Transfer Society**: Savoy, IL, USA.

TAO, Y.; ZHOU, B.; XIA, G.; WANG, F.; WU, Z.; FU, M. (2004) Exposure to L-Ascorbic Acid or α-Tocopherol Facilitates the Development of Porcine Denuded Oocytes from Metaphase I to Metaphase II and Prevents Cumulus Cells from Fragmentation. **Reproduction in Domestic Animals,** v. 39, n. 1, p. 52–57. doi:10.1046/j.1439-0531.2003.00478.x

TARÍN, J. J.; TROUNSON, A. O. (1993) Effects of Stimulation or Inhibition of Lipid Peroxidation on Freezing-Thawing of Mouse Embryos. **Biology of reproduction,** v. 49, n. 6, p. 1362–1368. PMID: 8286618

TATEMOTO, H.; OOTAKI, K.; SHIGETA, K.; MUTO, N. (2001) Enhancement of Developmental Competence After in vitro Fertilization of Porcine Oocytes by Treatment with Ascorbic Acid 2-O-Alpha-Glucoside During in vitro Maturation. **Biology of Reproduction**, v. 65, n. 6, p. 1800–1806. doi:10.1095/biolreprod65.6.1800

TATONE, C.; DI EMIDIO, G.; VENTO M.; CIRIMINNA R.; ARTINI, P. G. (2010) Cryopreservation and Oxidative Stress in Reproductive Cells. **Gynecological Endocrinology**. V. 26, n. 8, p. 563–567. doi: 10.3109/09513591003686395

TILLY, J. L.; TILLY, K. I. (1995) Inhibitors of Oxidative Stress Mimic the Ability of Follicle-Stimulating Hormone to Suppress Apoptosis in Cultured Rat Ovarian Follicles. **Endocrinology,** v. 136, n. 1, p. 242–252. doi:10.1210/endo.136.1.7828537

TOLBERT, B. M.; DOWNING, M.; CARLSON, R. W.; KNIGHT, M. K.; BAKER, E. M. (1975) Chemistry and Metabolism of Ascorbic Acid and Ascorbate Sulfate. **Annals of the New York Academy of Sciences**, v. 258, p. 48–69.

VEAL, E.A., DAY, A.M., MORGAN, B.A. (2007) Hydrogen Peroxide Sensing and Signaling. **Molecular Cell.** v. 26, n. 1, p. 1-14. doi: 10.1016/j.molcel.2007.03.016

WANG, X.; PH, D.; FALCONE, T.; ATTARAN, M. (2002) Vitamin C and Vitamin E Supplementation Reduce Oxidative Stress – Induced Embryo Toxicity and Improve the Blastocyst. **Fertility and Sterility**, v. 78, n. 6, p.1272-1277.

WILSON, J. X. (2005) Regulation of Vitamin C Transport. **Annual Review of Nutrition**, v. 25, p. 105–25. doi:10.1146/annurev.nutr.25.050304.092647

Tables

Table 1. Effect of AA antioxidant on re-expansion and hatching rate of cryopreserved IVP bovine embryos.

Treatment AA (mM)	Embryos (n)	Re-expansion rates (%)			Hatching rates (%)		
		24h	48h	72h	24h	48h	72h
0.0	144	61.1 [c]	63.9 [b]	71.5 [b]	17.4 [a]	32.6 [b]	42.4 [b]
0.1	148	77.7 [a]	80.4 [a]	87.8 [a]	25.7 [a]	43.9 [ab]	56.1 [a]
0.3	141	68.1 [ab]	71.6 [ab]	79.4 [ab]	23.4 [a]	44.7 [a]	54.6 [ab]
0.5	145	67.6 [b]	71.7 [a]	75.2 [b]	17.2 [a]	34.5 [ab]	46.2 [ab]

[a,b] Within a column, different subscribed letters indicate difference ($P<0.05$).

Table 2. Effects of AA antioxidant on total cells number and DNA fragmentation of cryopreserved IVP bovine embryos.

Treatment	n	Embryo cells number	TUNEL positive cells	% DNA fragmentation
Control	79	132.3 ± 3.7[a]	12.4 ±0.6[a]	9.6 ±4.8[a]
0.1 mM AA	87	130.3 ± 3.5[a]	9.0 ±0.6[b]	7.1 ±4.6[b]

Data represent least-squares means ± SEM

[a,b] Values within a column with different superscripts differ significantly ($P<0.001$).

Figure

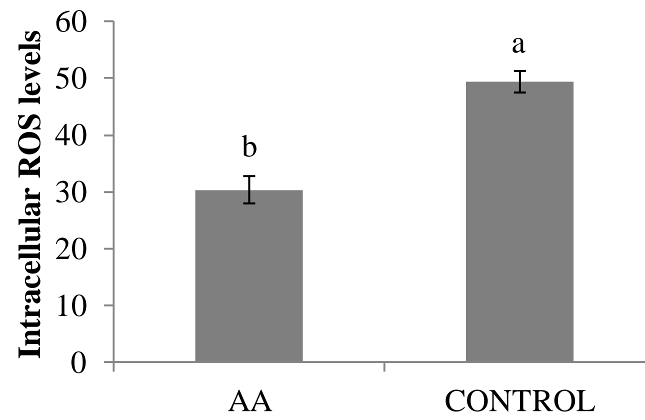

Figure 1. Intracellular ROS levels expressed as fluorescence intensity in cryopreserved embryos in the presence or absence of AA in slow freezing solutions. [a,b] Indicate difference ($P<0.001$) between groups.

CHAPTER 3

Do Dithiothreitol or Caspase-3 inhibitors improve the survival of in vitro produced bovine embryos during slow freezing?

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Abstract

In order to improve the cryotolerance of in vitro produced bovine embryos, two experiments were conducted to assess the effects of Dithiothreitol (DTT) and the caspase-3 inhibitor (z-DEVD-fmk) during cryopreservation. For the experiments, cumulus-oocyte complexes were obtained from ovaries of slaughterhouse cows; oocytes were in vitro matured, fertilized and cultured to day 7. Blastocyst and expanded blastocysts embryos were randomly assigned to be subjected to slow freezing. Embryos were slow-frozen in Hepes-TALP with 1.5 M Ethylene Glycol, 0.1 M Sucrose and DTT (0, 50, 100 and 200 µM) in experiment 1 or z-DEVD-fmk (0, 50, 100 and 200 µM) in experiment 2. Embryos were thawed and then cultured for 72 h. Re-expansion and hatching rates were recorded at 24, 48 and 72 h. There was no effect ($P > 0.05$) of treatment with DTT or z-DEVD-fmk on re-expansion or hatching rates at 24, 48 or 72 h post-thaw. This is the first report that indicates the use of antioxidant reagent (DTT) and the specific inhibitor of the apoptosis (z-DEVD-fmk) in the cryopreservation medium. In conclusion, DTT and z-DEVD-fmk treatments do not improve the survival of in vitro produced bovine embryos during slow freezing in the concentrations employed.

Key words: Caspase inhibitor; Cryopreservation; Dithiothreitol; Embryos.

Introduction

Cryopreservation is an important alternative for the preservation of embryos, however, the cells are subjected to different steps: (1) equilibration period; (2) cooling; (3) storage and (4) thawing (Faustino et al., 2010). These conditions can compromise cell recovery due the production of reactive oxygen species (ROS). The accumulation of these substances can induce massive cellular damage (Mignotte and Vayssiere, 1998) by releasing proteases, lipases and nucleases within the mitochondria, culminating in cell death (Fiers et al., 1999). Efforts to improve the survival rates of in vitro produced bovine embryos have been restricted primarily to changes in maturation and culture systems (Block and Hansen, 2007; Block et al., 2009; Pryor et al., 2009; Ruiz et al., 2014; Zolini et al., 2015; Block et al., 2015). Therefore, there is little information available about modifying cryopreservation solutions.

Dithiothreitol (DTT) is a small- molecule redox reagent. At low concentrations, DTT stabilizes enzymes that possess free sulfhydryl groups (Klonne and Johnson, 1988) and by this way protects the cells from apoptose. The environment of culture is an important determinant of continued embryonic development. Experiments using DTT have revealed that some of the actions of heat shock on development involve ROS production in a stage-specific manner and that ROS are important to trigger the induction of the apoptotic cascade. Induced apoptosis was blocked or greatly reduced by addition of DTT to culture medium (De Castro e Paula and Hansen, 2008). In others studies were also showed DTT actions, DTT protects cells from apoptosis by precluding the phosphorylation of protein tyrosine residues (Watson et al. 1996); reduces the thiol-oxidative damage caused by diamide in zygotes and blastocysts (Liu et al., 1999); increases fertilization rate, the number of cells post-insemination and potentiate the oocytes to develop to blastocysts (Tarín et al., 1998). However, the mode of action of DTT in protection of cells during cryopreservation has not been demonstrated.

Apoptosis has traditionally been considered an irreversible process with caspase activation committing the cell to death; however, previous studies have shown that inhibition of apoptosis responses with the caspase-3 inhibitor, z-DEVD-fmk, exacerbates effects of heat shock on embryo development (Paula-Lopes and Hansen, 2002) as well as the DTT completely blocked the induction of apoptosis

caused by heat shock (De Castro e Paula and Hansen, 2008). z-DEVD-fmk, a specific inhibitor of group II caspases, blocks the induction of apoptosis and DNA fragmentation in the oocyte and preimplantation embryos (Paula-Lopes and Hansen, 2002; Roth and Hansen, 2004; Jousan and Hansen, 2007). It is likely that the use of these two substances in cryopreservation medium neutralizes excessive ROS production preventing it from damaging the cellular structure of embryos submitted to freezing protocols.

For the current studies, two hypotheses were raised: 1) IVP embryos are highly susceptive to cryopreservation processes; therefore, modification of freezing medium by the addition of DTT protects cells by the reduction of ROS and consequently improves re-expansion and hatching rates post-thaw; 2) The addition of z-DEVD-fmk in the freezing medium, blocks the action of group II caspase to apoptosis inhibition improving re-expansion and hatching rates post-thaw.

Materials and Methods

All experimental procedures were approved by the Ethic Commission in use of Production Animals of Universidade Federal de Viçosa, Brazil (process number 41/2014).

Experimental Design

Experiment 1. Effect of DTT on re-expansion and hatching rate of cryopreserved IVP bovine embryos.

Bovine embryos were produced by in vitro fertilization of oocytes obtained from abattoir-collected ovaries. Immature cumulus oocyte complexes (COC's) were obtained by slicing follicles. Presumptive zygotes, 18-20 h post-insemination were placed in groups of 25-30 in 50 µL drops of synthetic oviduct fluid-bovine embryo 1 (SOF-BE1 – Fields et al., 2011). At Day 7 after insemination, the embryos at the blastocyst and expanded blastocyst stage evaluated as Grade 1 and 2 (according to the IETS manual; Stringfellow and Seidel, 1998) were harvested from culture. Embryos were subjected to controlled-rate freezing following equilibration for 10 min in freezing medium (Hepes-Tyrode's Albumin Lactate Pyruvate (Hepes-TALP) with 1.5 M ethylene glycol, 0.1 M Sucrose) and different Dithiothreitol (DTT;

D5545 Sigma Aldrich, St. Louis, MO, USA) concentrations 0 µM, 50 µM, 100 µM and 200 µM (in Annex). After exposure, the embryos were loaded in groups of six to 10 per straw of 0.25 mL. The experiment was replicated 9 times; harvested embryos (243) were used to determine re-expansion and hatching rates of bovine embryos during 24, 48 and 72 h post-thaw. There were between 56 and 63 embryos for each treatment.

Experiment 2. Effect of Caspase-3 inhibitor (z-DEVD-fmk) on re-expansion and hatching rate of cryopreserved IVP bovine embryos.

Bovine embryos were produced by in vitro fertilization of oocytes obtained from abattoir-collected ovaries. COC's were obtained by slicing follicles. Presumptive zygotes, 18-20 h post-insemination were placed in groups of 25-30 in 50 µL drops of SOF-BE1. At Day 7, the embryos at the blastocyst and expanded blastocyst stage (Grade 1 and 2; IETS; Stringfellow and Seidel, 1998) were harvested from culture. Embryos were subjected to controlled-rate freezing following equilibration for 10 min in freezing medium (Hepes-TALP with 1.5 M ethylene glycol, 0.1 M Sucrose) and different Caspase-3 inhibitor (z-DEVD-fmk; FMK004 R&D System, MN, USA) concentrations 0 µM, 50 µM, 100 µM and 200 µM (in Annex). After exposure, the embryos were loaded in groups of six to 10 per straw of 0.25 mL. The experiment was replicated 7 times; harvested embryos (227) were used to determine re-expansion and hatching rates of bovine embryos during 24, 48 and 72 h post-thaw. There were between 55 and 59 embryos for each treatment.

In vitro Embryo Production

Bovine embryos were produced by in vitro fertilization of oocytes obtained from abattoir-collected ovaries. All procedures for embryo production followed techniques previously described by Fields et al. (2011). COC's were obtained by slicing follicles from 2 to 10 mm in diameter on the surface of ovaries into a beaker containing oocyte washing medium (Mofa, Verona, WI, USA). Groups of 10 COC's were matured in 50 µL droplets of oocyte maturation medium [tissue culture medium-199 with Earle's salts (Invitrogen, Carlsbad, CA, USA), 10 % (v/v) bovine steer serum, 2 µg/mL estradiol 17-β, 20 µg/mL bovine follicle stimulating hormone (Bioniche Life Sciences, Ontario, Canada), 22 µg/mL sodium pyruvate, 50 µg/mL

gentamicin sulfate and 1 mM GlutaMAX™ (Life Technologies, Carlsbad, CA, USA)] covered with mineral oil for 20 hours at 38.5 °C and in a humidified atmosphere of 5 % (v/v) CO_2. Following maturation, COC's were washed once in Hepes-TALP (Caisson Laboratories, Inc., Logan, UT, USA; Parrish et al., 1986) and then transferred in groups of 200-300 to 35 mm Petri dishes containing 1.7 mL of in vitro fertilization-TALP (Caisson Laboratories, Inc., Logan, UT, USA; Parrish et al., 1986) and 120 µL of a mixture of 0.5 mM penicillamine, 0.25 mM hypotaurine and 25 µM epinephrine in 0.9% (w/v) NaCl and fertilized with 1 x 10^6 /mL spermatozoa purified from a pool of frozen-thawed conventional semen from three bulls using Isolate® separation gradient (Irvine Scientific, Santa Ana, CA, USA) for 18 - 20 h at 38.5 °C in a humidified atmosphere of 5% (v/v) CO_2. Mature oocytes were denuded after fertilization by vortexing in 600 µL Hepes-TALP containing 10,000 U/mL hyaluronidase and placed in groups of 25-30 in 50 µL microdrops of SOF-BE1 (Fields et al., 2011) covered with mineral oil at 38.5 °C in a humidified atmosphere of 5 % O_2 and 5 % CO_2 and 90% N_2. At Day 7 after insemination, embryos were harvested and subjected to slow freezing.

Slow-freezing procedure

Blastocyst and expanded blastocysts grade 1 and 2 according to the guidelines of the International Embryo Transfer Society (IETS; Stringfellow and Seidel, 1998) were harvested from culture on Day 7 (Day 0 = day of in vitro fertilization), washed three times in Hepes-TALP at room temperature and then transferred directly into the freezing medium (Hepes-TALP plus 1.5 M ethylene glycol (Sigma Aldrich, St. Louis, MO, USA) and 0.1 M Sucrose (Sigma Aldrich, St. Louis, MO, USA)] with dithiothreitol (DTT), or caspase-3 inhibitor (z-DEVD-fmk) treatments. Embryos were loaded in groups of six to 10 per straw of 0.25 mL with three columns of freezing medium separated by 5 mm air columns and embryos were loaded into the middle column of straws and allowed to equilibrate for 10 min.

The embryos into straws were then placed into the chamber of a programmable freezing machine (CryoLogic Freeze Control® CL-5500, Victoria, Australia) set at -6.0 °C and after 2 min, straws were seeded by touching the top column of medium with a cotton swab dipped in liquid nitrogen. The straws were

maintained at this temperature for 10 min, and then cooled to -32 °C at 0.5 °C per min and then held for 10 min prior to being plunged into liquid nitrogen (-196 °C).

Post Thaw Embryo Survival

At the time of thawing, embryos were removed from liquid nitrogen and held in the air at room temperature for 5 seconds, followed by immersion into a water-bath at 30 °C for 20 seconds. Embryos were washed three times in Hepes-TALP at room temperature and cultured for 72 h in groups of 6-10 in 25 μL microdrops of SOF-BE1 supplemented with 10% fetal bovine serum overlaid with mineral oil at 38.5 °C in a humidified atmosphere of 5 % (v/v) CO_2, 5 % (v/v) O_2 and 90% N_2. Re-expansion and hatching rates (including both hatching and hatched embryos) were recorded at 24, 48 and 72 h post-thaw.

Statistical analysis

For data analysis, the Statistical Analysis System (SAS, version 9.3; SAS Institute Inc., Cary, NC, USA) was used. Re-expansion and hatching rates were evaluated by logistic regression considering treatment and replication effects. For discussion of results only the effect of treatment was considered. Significant level adopted was $\alpha = 0.05$.

Results

Experiment 1. Effect of DTT on re-expansion and hatching rate of cryopreserved IVP bovine embryos.

The results of IVP embryos cryopreserved in slow-freezing medium with or not DTT antioxidant are show in the Table 1. The addition of DTT during cryopreservation did not affect re-expansion and hatching rates at 24, 48 and 72 h post-thaw compared with embryos that were not treated with DTT. Supplementation with 200 μM DTT increased ($P<0.05$) hatching rates at 72 h compared with 50 μM group, but did not differ from control and 100 μM DTT treatments.

Experiment 2. Effect of Caspase-3 inhibitor (z-DEVD-fmk) on re-expansion and hatching rate of cryopreserved IVP bovine embryos.

Data for the re-expansion and hatching rates of bovine embryos cryopreserved in slow freezing medium with z-DEVD-fmk addition are presented in Table 2. There was no effect (P>0.05) of z-DEVD-fmk on re-expansion and hatching rates during 24, 48 and 72 h post-thaw.

Discussion

The protection against ROS can be provided by enzymatic degradation, removal by antioxidants and molecular repair. In the present study, DTT was used as an antioxidant because it is a strong reducing agent, it is used to reduce disulfide bonds quantitatively and maintain monothiols in the reduced state (Cleland, 1964), contains two thiol groups for sequential thiol-disulfide exchange reactions for direct scavenging of ROS as well as regeneration of reduced glutathione from oxidized glutathione (Rothwarf and Scheraga 1992). In this study, the addition of DTT in slow-freezing medium had no effects on re-expansion and hatching rates, probably that concentrations used were not sufficient to exacerbate the oxidation caused by cryopreservation, harming the developmental potential of embryos. However, Liu et al. (1999) demonstrated the effective against thiol-oxidative damage using the same concentrations of DTT in zygotes and blastocysts. They induced apoptosis in mouse zygotes and blastocysts by Diamide, a SH-specific oxidant that can inhibit embryo development and provoke cell cycle arrest, apoptosis, and/or cell death. On the other hand, when Tarín et al., (1998) used 50 and 500 μM of DTT, fertilization rate was increased and number of cells at 81 h post-insemination also, both age-associated fragmentation at 24 h post-insemination and decreased potential of oocytes for development to the blastocyst stage were prevented by culturing oocytes in the presence of 50 μM of DTT. Castro e Paula and Hansen (2008) demonstrated that the heat-shock induced apoptosis was blocked by culture in low oxygen (5% oxygen) and by addition of DTT to culture medium. The thermoprotective effect of DTT depended on the stage of development, for the two-cell embryo the degree of inhibition of blastocyst development caused by heat shock in the presence of DTT (500 μM) was 77.3% compared to 95.5% in the absence of DTT, in contrast for the Day 5 was 26.6% compared to 70.8% of control embryos.

Redox imbalance is an important apoptotic pathway that could occur during cryopreservation and embryo development. The capacity for blastocysts to undergo further development is related to degree of group II caspase activity (ie., caspase-2, -3 and -7; Jousan et al., 2008). The caspases are intracellular proteases that exist in the cells as inactive precursors. Caspase activation can occur by two pathways: the extrinsic (receptor-mediated) and intrinsic (stress-mediated). In both pathways, initiator caspases (caspase-2, 8 and/or 10) activates other effector caspases (caspase-3, 7 and 6) resulting in membrane blebbing, nuclear and chromatin condensation and fragmentation, shrinking of the cell, mitochondrial disintegration and formation of apoptotic bodies (budding off cellular portions) which ultimately leads to cell death (Xu et al., 2001; Danial and Korsmeyer, 2004; Hussein, 2005; Faustino et al., 2011). There are evidence that mouse embryos express mRNA of caspases 2, 3, 6, 7, and 12 (Exley et al., 1999), apoptosis genes (survivin, Fas, Hsp 70 and caspase-3) were expressed in frozen-thawed bovine blastocysts (Park et al., 2006) and that apoptosis is dependent on embryonic developmental stage after standard culture (Matwee et al., 2000). To investigate the relationship between cryopreservation and apoptosis, z-DEVD-fmk was used in slow freezing medium in different concentrations 50, 100 and 200 µM to treat bovine embryos. There was no statistically significant difference in the percentage of re-expansion and hatching during 24, 48 and 72 h post-thaw between the control and those treated with caspase inhibitors; Effective action of z-DEVD-fmk has already been evidenced in other studies but not in embryo cryopreservation. Paula-Lopes and Hansen (2002), demonstrated that z-DEVD-fmk, blocks the heat induced caspase activity and prevents the increase in TUNEL-positive blastomeres observed after heat shock. This fact was confirmed years later by Roth and Hansen (2004) and Jousan and Hansen (2007) in the oocyte and preimplantation embryo.

Conclusion

This study provides the first evidence of DTT and z-DEVD-fmk impacts when added in slow-freezing medium. In the concentrations employed, DTT and z-DEVD-fmk do not improve the survival of bovine cryopreserved in vitro produced embryos.

Acknowledgments

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References

BLOCK, J.; and HANSEN, P.J. (2007) Interaction Between Season and Culture with Insulin-Like Growth Factor-1 on Survival of in vitro Produced Embryos Following Transfer to Lactating Dairy Cows. **Theriogenology**. v. 67, p. 1518–1529.

BLOCK, J.; BONILLA, L.; HANSEN, P.J. (2009) Effect of Addition of Hyaluronan to Embryo Culture Medium on Survival of Bovine Embryos in vitro Following Vitrification and Establishment of Pregnancy After Transfer to Recipients. **Theriogenology.** 71:1063–1071.

BLOCK, J.; ZOLINI, A. M.; CARRASCAL-TRIANA E. L.; RUIZ, A.; HANSEN, P.; TORRES C. A. A. (2015) 34 Effects Of L-Carnitine And Trans-10,Cis-12 Conjugated Linoleic Acid Supplementation During Maturation on Development And Cryotolerance of Bovine Embryos Produced in vitro. Reproduction, **Fertility and Development** v. 28 (2) p. 147-147 doi:10.1071/RDv28n2Ab34

CLELAND, W. W. (1964) Dithiothreitol, a New Protective Reagent for SH Groups. **Biochemistry,** v.3 (4), p. 480–482. doi: 10.1021/bi00892a002

DANIAL, N.N.; KORSMEYER, S.J. (2004) Cell Death: Critical Control Points. **Cell,** v. 23, n. 116 (2) p. 205-19.

DE CASTRO E PAULA, L. A.; HANSEN, P. J. (2008) Modification of Actions of Heat Shock on Development and Apoptosis of Cultured Preimplantation Bovine Embryos by Oxygen Concentration and Dithiothreitol. **Molecular Reproduction and Development**, v.75, p. 1338–1350. doi:10.1002/mrd.20866

EXLEY, G. E.; TANG, C.; MCELHINNY, A. S.; WARNER, C. M. (1999) Expression of Caspase and BCL-2 Apoptotic Family Members in Mouse Preimplantation Embryos. **Biology of Reproduction**, v.61, p. 231-239. doi: 10.1095/biolreprod61.1.231

FAUSTINO, L. R.; SANTOS, R. R.; SILVA, C. M.; PINTO, L. C.; CELESTINO, J. J.; CAMPELLO, C. C.; FIGUEIREDO, J. R.; RODRIGUES, A. P. (2010) Goat and Sheep Ovarian Tissue Cryopreservation: Effects on the Morphology and Development of Primordial Follicles and Density of Stromal Cell. **Animal Reproduction Science**, v. 122, p. 90-97. doi: 10.1016/j.anireprosci.2010.08.001

FAUSTINO, L. R.; CARVALHO, A. A.; DA SILVA, C. M. G.; FIGUEIREDO, J. R.; RODRIGUES A. P. R. (2011) Criopreservação de Tecido Ovariano: Limitações e Perspectivas para a Preservação da Fertilidade de Fêmeas. **Acta Scientiae Veterinariae**. v. 39, n. 3:972.

FIELDS, S. D.; HANSEN P. J.; EALY A. D. (2011) Fibroblast Growth Factor Requirements for in vitro Development of Bovine Embryos. **Theriogenology**, v.75, p. 1466–1475. doi: 10.1016/j.theriogenology.2010.12.007

FIERS, W.; BEYAERT, R.; DECLER, W.; PETER, V. (1999) More than one Way to Die: Apoptosis, Necrosis and Reactive Oxygen Damage. **Oncogene**, v. 18, p. 7719-7730. doi: 10.1038/sj.onc.1203249

HUSSEIN M.R. (2005) Apoptosis in the Ovary: Molecular Mechanisms. **Human Reproduction Update**, v. 11 n. (2), p. 162-177. doi: 10.1093/humupd/dmi001

JOUSAN, F. D.; HANSEN, P. J. (2007) Insulin-Like Growth Factor-I Promotes Resistance of Bovine Preimplantation Embryos to Heat Shock Through Actions

Independent of its Anti-Apoptotic Actions Requiring PI3K Signaling. **Molecular Reproduction and Development,** v.74, p. 189-196. doi: 10.1002/mrd.20527

JOUSAN, F. D.; OLIVEIRA, L. J.; HANSEN, P. J. (2008) Short-Term Culture of in vitro Produced Bovine Preimplantation Embryos with Insulin-Like Growth Factor-I Prevents Heat Shock-Induced Apoptosis Through Activation of the Phosphatidylinositol 3-Kinase/Akt Pathway. **Molecular Reproduction and Development,** v.75, p. 681–688. doi: 10.1002/mrd.20830

KLONNE D. R.; JOHNSON D. R. (1988) Enzyme Activity and Sulfhydryl Status in Rat Renal Cortex Following Mercuric Chloride and Dithiothreitol Administration. **Toxicology Letters,** v. 42, p. 199-205. doi:10.1016/0378-4274(88)90078-1

LIU, L.; TRIMARCHI, J. R.; KEEF, D. L. (1999) Thiol Oxidation-Induced Embryonic Cell Death in Mice is Prevented by the Antioxidant Dithiothreitol. **Biology of Reproduction**, V.61, p. 1162–1169. doi: 10.1095/ biolreprod61.4.1162

MATWEE, C.; BETTS, D.H.; KING, W. A. (2000) Apoptosis in the Early Bovine Embryo. **Zygote**. v.8, p.57-68. doi: 10.1017/S0967199400000836

MIGNOTTE, B.; VAYSSIERE, J. (1998) Mitochondria And Apoptosis. **European Journal of Biochemistry**, v. 252, p. 1-15. doi: 10.1046/j.1432-1327.1998.2520001.x

PARK, S. Y.; KIM, E. Y.; CUI, X. S.; TAE J. C.; LEE, W. D.; KIM, N. H.; PARK, S. P.; LIM, J. H. (2006) Increase in DNA Fragmentation and Apoptosis-Related Gene Expression in Frozen-Thawed Bovine Blastocysts. **Zygote,** v.14, p.125-131. doi: 10.1017/S0967199406003649

PARRISH, J. J.; SUSKO-PARRISH, J. L.; LEIBFRIED-RUTLEDGE, M. L. CRITSER, E. S.; EYESTONE, W. H.; FIRST N. L. (1986) Bovine in vitro

Fertilization with Frozen-Thawed Semen. **Theriogenology,** v.25, p. 591–600. doi: 10.1016/0093-691X(86)90143-3

PAULA-LOPES, F. F.; HANSEN, P. J. (2002) Apoptosis is an Adaptive Response in Bovine Preimplantation Embryos That Facilitates Survival After Heat Shock. **Biochemical and Biophysical Research Communications**, v.295, p. 37-42. doi: 10.1016/S0006-291X(02)00619-8

PRYOR, J. H.; TRANT, J. A.; PONCHIROLLI-SCHNEIDER, C. B.; LOONEY, C. R.; LONG, C. R.; FORREST. D.W. (2009) 110 The use of Forskolin and its Effect on in vitro Produced Brahman-Sired Embryos Submitted to Slow Cool Freezing or Vitrification. **Reproduction, Fertility and Development.** v. 22 p. 214–214.

ROTH, Z.; HANSEN, P.J. (2004) Involvement of Apoptosis in Disruption of Developmental Competence of Bovine Oocytes by Heat Shock During Maturation. **Biology of Reproduction,** v.71, p. 1898-1906. doi: 10.1095/ biolreprod.104.031690

ROTHWARF, D. M.; SCHERAGA, H. A. (1992) Equilibrium and Kinetic Constants for the Thiol-Disulfide Interchange Reaction Between Glutathione and Dithiothreitol. **Proc Natl Acad Sci U S A,** v.89, p.7944-7948. PMCID: PMC49831

RUIZ, A.; HANSEN, P.; BLOCK, J. (2015) 53 Effect of Addition of L-Carnitine and Conjugated Linoleic Acid During Bovine Embryo Culture on Cryosurvival, Lipid Content. Reproduction, **Fertility and Development**, v. 27, p. 119–120. doi:10.1071/RDv27n1Ab53

STRINGFELLOW D. A.; SEIDEL S. M. (1998) ‘Manual of the International Embryo Transfer Society: A Procedural Guide and General Information for the Use of Embryo Transfer Technology Emphasizing Sanitary Procedures’. **International Embryo Transfer Society**: Savoy, IL, USA.

TARÍN, J. J.; TEN, J.; VENDRELL, F.J.; CANO, A. (1998) Dithiothreitol Prevents Ageassociated Decrease in Oocyte/Conceptus Viability in vitro. **Human Reproduction**, v.13, p. 381–386. doi: 10.1093/humrep/13.2.381

XU, J.; CHEUNG, T.; CHAN, S. T.; PAK-CHUNG, M. D.; YEUNG, W. S. (2001) The Incidence of Cytoplasmic Fragmentation in Mouse Embryos in vitro is not Affected by Inhibition of Caspase Activity. **Fertility and Sterility**. v.75, n5, p. 986- 991. doi: 10.1016/S0015-0282(01)01687-9

WATSON, R. W. G.; ROTSTEIN, O. D.; NATHENS, A. B.; ALAN, P. B.; Dackiw.;. Marshall, J. C. (1996) Thiol-Mediated Redox Regulation of Neutrophil Apoptosis. **Department of Surgery,** University of Toronto, Canada. V.120, p. 150–158. doi: 10.1016/S0039-6060(96)80282-0

ZOLINI, A. M.; CARRASCAL-TRIANA E. L.; RUIZ, A.; PENITENTE-FILHO J.M.; HANSEN, P.; TORRES C. A. A; BLOCK, J. (2015) 163 Effects of Serum and L - Carnitine on Development and Cryotolerance of Bovine Embryos Produced in vitro. Reproduction, **Fertility and Development,** v. 28(2), p. 211–212.

Tables

Table 1. Effect of DTT on re-expansion and hatching rate of cryopreserved IVP bovine embryos.

Treatment DTT (μM)	Embryos (n)	Re-expansion rates (%)			Hatching rates (%)		
		24h	48h	72h	24h	48h	72h
0	63	66.7	73.0	76.2	15.9	33.3	46.0 [ab]
50	61	60.7	67.2	67.2	13.1	21.3	29.5 [b]
100	56	69.6	71.4	78.6	7.1	19.6	32.1 [ab]
200	63	73.0	77.8	82.5	22.2	38.1	50.8 [a]

[a,b] Within a column, different subscribed letters indicate difference ($P<0.05$).

Table 2. Effect of Caspase-3 inhibitor (z-DEVD-fmk) on re-expansion and hatching rate of cryopreserved IVP bovine embryos.

Treatment z-DEVD-fmk (μM)	Embryos (n)	Re-expansion rates (%)			Hatching rates (%)		
		24h	48h	72h	24h	48h	72h
0	59	69.5	79.7	78.0	20.3	39.0	45.8
50	55	74.5	81.8	83.6	20.0	45.5	52.7
100	57	71.9	80.7	80.7	29.8	40.4	45.6
200	56	76.8	85.7	85.7	21.4	44.6	60.7

($P>0.05$)

CONCLUSION

This is the first report that used AA and DTT antioxidants or z-DEVD-fmk a specific inhibitor of the apoptosis in the cryopreservation medium of in vitro produced bovine embryos. The addition of AA (0.1 mM) in slow-freezing medium improves the cryosurvival of in vitro produced bovine embryos, reduces intracellular reactive oxygen species levels and DNA fragmentation. DTT and z-DEVD-fmk treatments have no effect on post-thaw embryo survival in the concentrations employed.

ANNEX

- **ASCORBIC ACID (AA)**

1 M = 198,11 mg/mL

Stock solution: 0.09905 g of AA dilute in 5 mL Hepes-TALP
(Filter – Aliquots of 250 µL)

0.1mM AA: 100mM x X =0.1mM x5mL
X=0.005mL

0.3mM AA: 100mM x X =0.3mM x5mL
X=0.015mL

0.5mM AA 100mM x X =0.5mM x5mL
X=0.025mL

- **DITHIOTHREITOL (DTT)**

1 M = 154.25 g/Mol

50 µM = 0.00005 Mol

(1000x)=0.5 Mol

Stock solutions: 1M . V = 0.05M x 160 µL
V=8 µL; 10 Aliquots (16 µL)

50µM DTT 0.05 M . V = 0.00005M x 2000 µL
V=2 µL

100µM DTT 0.05 M . V = 0.0001M x 2000 µL
V=4 µL

200µM DTT 0.05 M . V = 0.0002M x 2000 µL
V=8 µL

- **CASPASE-3 INHIBITOR (z-DEVD-fmk)**

NW: 668 Da

1mg

Reconstitution: 75 µL of DMSO to vial yield a 20 mM.

Stock solution

1. Dilute 675 µL of freeze solution in z-DEVD-fmk reconstituted.

   750 µL; Aliquots (92 µL) and store at -20 °C.

2. Dilute 675 µL of freeze solution with 75 µL of DMSO.

   750 µL; Aliquots (120 µL) and store at -20 °C.

1:10 = 200 µL

1:20 = 100 µL

1:40 = 50 µL