UNIVERSIDADE ESTADUAL PAULISTA
FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS
CÂMPUS JABOTICABAL

USO DE ASTAXANTINA EM CULTIVO *IN VITRO* DE EMBRIÕES BOVINOS E EFEITOS NA CRIOPRESERVAÇÃO

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Médica Veterinária

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IMPACTO POTENCIAL DESTA PESQUISA

O impacto desta pesquisa consiste determinar que embriões bovinos cultivados *in vitro* suplementados com o antioxidante astaxantina atravesse as etapas da criopreservação com menos crioinjúrias, seguindo para a posterior fase de reaquecimento e consequentemente trazendo resultados satisfatórios na transferência embrionária. Este estudo contribui para futuros projetos direcionados a etapa de criopreservação, já que o embrião adquire uma fragilidade estrutural inerente após este processo e os mecanismos de defesa próprio dos embriões são insuficientes para proteger suas delicadas estruturas celulares.

POTENTIAL IMPACT OF THIS RESEARCH

The impact of this research is to determine that *in vitro* cultured bovine embryos supplemented with the antioxidant astaxanthin go through the stages of cryopreservation with less cryoinjury, moving on to the subsequent rewarming phase and consequently bringing satisfactory results in embryonic transfer. This study contributes to future projects aimed at the cryopreservation stage, since the embryo acquires an inherent structural fragility after this process and the embryos' own defense mechanisms are insufficient to protect their delicate cellular structures.

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CERTIFICADO DE APROVAÇÃO

TÍTULO DA DISSERTAÇÃO: O USO DA ASTAXANTINA NO MEIO EM CULTIVO *IN VITRO* DE EMBRIÕES BOVINOS E EFEITOS NA CRIOPRESERVAÇÃO

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AUTHOR'S CURRICULUM DATA

FERNANDA SAID AIDAR – Born in São Paulo on September 17th, 1980. She graduated in Veterinary Medicine on December 9th, 2002 from Universidade do Oeste Paulista - Presidente Prudente - SP, specialized in Reproduction and Production Animals on June 28th, 2004 under the guidance of Prof. Dr Marcelo Marcondes Seneda. Since 2006, she has been carrying out activities in *in vitro* fertilization for cattle. She developed team training, franchise management in laboratories nationally and internationally and improvement of commercial techniques in the research for better results. She is currently working in an IVF company for cattle named AB Europe in Scotland as their IVP Laboratory Manager, as well as a Master's student in Animal Reproduction at the Veterinary Medicine Program at the São Paulo State University "Júlio de Mesquita Filho", under the supervision of Prof. Dr. Lindsay Unno Gimenes.

From 08/2020 to 02/2022 she had a scholarship provided by Capes (financial code 001) for the development of the project.

"...whoever really gets involved with this work will be led to a path that will demand the most from it, but will also be given the most".

Bert Hellinger

Dedication

To my mum Sossô and my aunts San and Sé who supported and encouraged me to return to my studies after so many years away from the University.

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To my parents for giving me the opportunity to study, to invest in my academic career, for believing that studying away from home would be as difficult as any new challenge, but that this would also bring my personal evolution as a gift.

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Câmpus de Jaboticabal

CEUA – COMISSÃO DE ÉTICA NO USO DE ANIMAIS

DECLARAÇÃO

Declaramos que o trabalho de pesquisa intitulado **"Adequação de protocolos no uso da dimetilformamida na criopreservação de embriões bovinos produzidos *in vitro*"**, sob a responsabilidade da Profª Drª Lindsay Unno Gimenes e Certificado CEUA protocolo nº 5499/20, aprovado em reunião ordinária em 11 de fevereiro de 2021, apresentou alteração no título do projeto, para: **"O USO DA ASTAXANTINA NO MEIO DO CULTIVO IN VITRO EXERCENDO EFEITOS POSITIVOS NA CRIOPRESENVAÇÃO DE EMBRIÕES BOVINOS"**. Além disso, será realizado a substituição da molécula Dimetilformamida por Astaxantina.

Jaboticabal, 20 de fevereiro de 2022.

Fabiana Pilarski

Prof.ª Dr.ª Fabiana Pilarski
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ABREVIATION LIST

AI	Apoptotic Index
AST	Astaxanthin
ATP	Adenosine Triphosphate
BME	Amino Acids Solution
BSA	Albumin from Bovine Serum
CEUA	Ethics Committee on the Use of Animals
C=O	Keto
CO_2	Carbon Dioxide
COC	Cumulus-Oocyte Complex
DMSO	Dimethyl Sulfoxide
DPBS	Dulbecco's Phosphate Buffered Saline
dUTP	Triphosphated Deoxyuridine Nucleotides
EG	Ethylene Glycol
FCS	Fetal Calf Serum
FSH	Follicle-stimulating Hormone
FITC	Fluorescein Isocyanate
GL	Glycerol
hCG	Human Chorionic Gonadotropin
HM	Holding Medium
H_2O_2	Hydrogen Peroxide
IETS	International Embryo Technology Society
IN	Necrotic Index
IVC	*In Vitro* Culture Medium
IVF	*In Vitro* Fertilization Medium
IVM	*In Vitro* Maturation Medium
LPO	Lipid Peroxidation
M	Molar
MDA	Malondialdehyde
MEM	Non-essential Amino Acids Solution
NaOH	Sodium Hydroxide
$NaHCO_3$	Bicarbonate Medium
N_2 L	Liquid Nitrogen

O_2	Oxygen
O_{2^-}	Anion Superoxide
–OH	Hydroxyl
PDO	Propanediol
IVEP	*In Vitro* Embryo Production
PVA	Polyvinylpyrrilidone
PZM-3	Medium Defined for Swine Embryos
RO	Alkoxyl Radicals
ROO	Interceptor of Peroxyl
ROS	Reactive Oxygen Species
SOF	Synthetic Oviductal Fluid
TALP-SPERM	Tyrode’s Albumin Lactate Pyruvate
TBA	Thiobarbituric Acid
TBARS	Thiobarbituric Acid Reactive Substance
TCA	Trichloroacetic Acid Solution
TdT	Terminal Deoxynucleotidyl Transferase
µg/L	Micrograms / Liter
µM	Micromolar
UV	Ultra Violet
VS1	Vitrification Solution 1
VS2	Vitrification Solution 2
xG	G force

USO DE ASTAXANTINA EM CULTIVO *IN VITRO* E EFEITOS SUBSEQUENTES NA CRIOPRESERVAÇÃO DE EMBRIÕES BOVINOS

RESUMO –O objetivo deste trabalho é avaliar se a adição de astaxantina (AST) ao meio de cultivo *in vitro* (CIV) pode exercer efeitos positivos em embriões bovinos após vitrificação. A AST é um carotenoide com altas propriedades antioxidantes, devido a uma estrutura molecular única contendo ligações duplas conjugadas e grupos hidroxila (–OH) e ceto (C=O), que contribuem para eliminar EROs e bloquear a peroxidação lipídica (LPO). Os presumíveis zigotos foram atribuídos 22 horas após a fertilização *in vitro* em um dos seguintes tratamentos: G-CONT) Controle, G-DMSO) dimetilsulfóxido (1 µl de DMSO / 999 µl de meio CIV), G-AST 0,8) 0,8 nM de AST, G- AST 3) 3 nM de AST e G-AST 13) 13 nM de AST. Apenas blastocistos expandidos (BX) grau I no 7º dia de desenvolvimento foram vitrificados (n = 92, 95, 86, 99 e 95/grupo, respectivamente). Os embriões foram avaliados a fresco e vitrificados quanto à taxa de eclosão e reexpansão, viabilidade celular incluindo número total de células e apoptose pelo teste TUNEL, além dos níveis lipídicos de peroxidação dos meios de cultura com substâncias reativas ao ácido tiobarbitúrico (TBARS). Nossos resultados mostraram que não houve diferença devido à suplementação de AST na formação de blastocistos no D7. Entretanto, a adição de 0,8, 3 e 13 nM de AST ao meio CIV aumentou a taxa de expansão dos blastocistos após 24 h nos grupos G-AST 0,8 (100^{A}(100-100)); G-AST 3 (98^{A}(100-100)); G-AST 13 (100^{A}(100-100)) quando comparado ao G-CONT (93^{B}(100-100)) e G-DMSO ($94,3^{B}$(100-100)) e 48 h de cultura G-AST 0,8 (100^{A}(100-100)); G-AST 13 (100^{A}(100-100)) quando comparado ao G-CONT ($91,3^{B}$(83,3-100)); G-DMSO ($92,4^{B}$(80-100)); G-AST 3 ($95,5^{AB}$(100-100)), independente se o embrião era fresco ou vitrificado. A porcentagem de células apoptóticas nos embriões após vitrificação/aquecimento foi maior no G-AST 0,8 ($17,9^{A}$(13,5-21,4)); ($p<0,05$) comparado ao G-DMSO $9,7^{B}$(8,1-10,7) e G-AST 3 ($11,1^{B}$ (10,0-12,8); ($p<0,05$). TBARS não mostrou efeito da astaxantina sob a LPO quando adicionada ao meio da CIV, não houve diferenças entre os grupos para os embriões do G-CONT no 7º dia de estágio de desenvolvimento ($p > 0,05$). Em conclusão, a suplementação com AST no meio de cultivo controlou parcialmente os efeitos da scrioinjúrias nos embriões vitrificados, levando à formação de blastocistos com uma taxa comparativamente aumentada de embriões BX após procedimentos de aquecimento. Tomados em conjunto, este estudo fornece novas perspectivas na compreensão de como a AST poderia melhorar a competência de desenvolvimento de embriões bovinos vitrificados.

Palavras-chave: astaxantina, carotenoides, crioprotetores, espécies reativas de oxigênio.

THE USE OF ASTAXANTHIN IN *IN VITRO* CULTURE AND SUBSEQUENT EFFECTS ON CRYOPRESERVATION OF BOVINE EMBRYOS

ABSTRACT – The objective of this work is to evaluate whether the addition of astaxanthin (AST) to the *in vitro* culture medium (IVC) can exert positive effects on bovine embryos after vitrification. The AST is a carotenoid with high antioxidant properties, due a unique molecular structure containing conjugated double bonds, and hydroxyl (–OH) and keto (C=O) groups, which contribute to eliminate ROS and block lipid peroxidation (LPO). Presumable zygotes were assigned 22 hours after IVF in one of the following treatments: G-CONT) Control, G-DMSO) dimethyl sulfoxide (1 µl DMSO/999 µl IVC medium), G-AST 0.8) 0.8 nM of AST, G-AST 3) 3 nM of AST and G-AST 13) 13 nM of AST. Only grade I expanded blastocysts (XB) on 7th day of development were vitrified (n = 92, 95, 86, 99, and 95/group, respectively). Embryos were evaluated fresh and vitrified for hatching rate and re-expansion, also cell viability including the total number of cells and apoptosis by TUNEL test, in addition to peroxidation lipid levels of culture media with thiobarbituric acid reactive substances (TBARS). Our results showed that there was no difference due to AST supplementation in blastocyst formation on D7. However, the addition of 0.8, 3 and 13 nM of AST into the IVC medium increased the expansion rate of blastocysts after 24 h in the groups G-AST 0.8 (100^{A}(100-100)); G-AST 3 (98^{A}(100-100)); G-AST 13 (100^{A}(100-100)) when compared to G-CONT (93^{B}(100-100)) and G-DMSO (94.3^{B}(100-100)) and 48 h of culture G-AST 0.8 (100^{A}(100-100)); G-AST 13 (100^{A}(100-100)) when compared to G-CONT (91.3^{B}(83.3-100)); G-DMSO (92.4^{B}(80-100)); G-AST 3 (95.5^{AB}(100-100)), independent if the embryo was fresh or vitrified. The apoptotic cell percentage in the embryos after vitrification/warming was higher in the G-AST 0.8 (17.9^{A} (13.5-21.4); ($p<0.05$) compared to G-DMSO 9.7^{B}(8.1-10.7) and G-AST 3 (11.1^{B} (10.0-12.8); ($p<0.05$). TBARS showed no effect of astaxanthin on IVC medium LPO, no differences among the groups to the embryos of the G-CONT at the 7th day of developmental stage ($p > 0.05$). In conclusion, supplementation with AST to the culture medium partially controlled cryoinjury effects on the vitrified embryos, leading to formation of blastocysts with a comparatively increased rate of XB embryos after warming procedures. Taken together, this study provides new perspectives in understanding how AST could improve the developmental competence of vitrified bovine embryos.

Keywords: astaxanthin, carotenoids, cryoprotectants, reactive oxygen species.

CHAPTER 1 - GENERAL CONSIDERATIONS

1. INTRODUCTION

In vitro embryos are highly sensitive to the external environment. In the process of *in vitro* culture, changes in osmotic pressure, pH, gas phase environment, and temperature lead to the excessive accumulation of reactive oxygen species (ROS) and damage cells, reducing the quality of *in vitro* embryos (KANE, 2003, SHAHBAZI et al., 2016, SCIORIO; THONG; PICKERING, 2018). IVC environments for embryos suffer from higher oxygen concentrations (GUERIN; EL MOUATASSIM; MENEZO,2001) resulting in ROS accumulation and inhibition of embryonic development (JAMIL et al., 2020). In addition to that, oxidative stress during IVC can lead to apoptosis (YOUSEFIAN et al., 2021).

A multitude of key factors regulate the generation of ROS in the media and include various cellular metabolic reactions, oxygen concentration and general physicochemical parameters that may have a negative impact on physiology by inducing apoptosis (KHAZAEI; AGHAZ, 2017). Oxygen tension probably has important implications for the regulation of cell proliferation and differentiation during the early stages of embryo development. Although oxygen tensions during oviductal transit are higher (maximum 9% oxygen), but this is still far below the 20% oxygen which is present under atmospheric conditions (FISCHER; BAVISTER, 1993). During *in vitro* embryo production (IVEP), it is challenging to recreate a balanced oxidative environment due to the high oxygen tensions during manipulation (SIDI et al., 2022).

Oxidative stress during culture can be mitigated by using antioxidant supplementation. One of the antioxidants that has become popular during oocyte and embryo culture is astaxanthin (AST). It has been shown that AST supplementation during oocyte and embryo culture promotes oocyte maturation and developmental competence (YOUNG et al., 2001, KUROKI et al., 2013, DO et al., 2015, ISPADA et al., 2018, XIANG et al., 2021). Recently, AST is shown to exert the beneficial effect on mammalian reproduction, and can ameliorate the development defect of early embryos caused by harmful factors [DO et al., 2015, ISPADA et al., 2018; HE et al., 2022). AST is also proven to alleviate ROS

mediated apoptosis, and improve DNA methylation reprogramming (LI et al., 2015; KIM and KIM, 2018).

Therefore, the present study will be conducted to determine whether antioxidant AST at different concentrations may be beneficial for the development of oocytes up to the stage of morula and blastocyst when added during the IVC of bovine zygotes under defined condition of high O_2, mitigating the effects of cryoinjury in the freezing process.

2. LITERATURE REVIEW

2.1. Embryo culture

Some studies have suggested that the development of embryos depends on the quality of the oocytes from which they originate (LONERGAN et al., 2003; RODRIGUEZ; FARIN, 2004). However, there is also evidence that the environment to which embryos are exposed during culture can affect their quality and viability (VAN SOOM et al., 2002; YUAN et al., 2003).

Along the years, the harmful effect of oxidative injury on the development of mammalian embryos grown *in vitro* have been studied. Toxic effects of atmospheric oxygen concentration under normal culture conditions (5% CO_2 and 95% air; approximately 20% O_2) was replicated in hamster (BAVISTER, 1988, MCKIERNAN; BAVISTER, 1990), pig (WRIGHT, 1977), sheep (THOMPSON et al., 1990), and cow (THOMPSON et al., 1990, VOELKEL; HU, 1992). This reduction in development is probably due to oxidative stress, since there is the addition of sequestered free radicals (NODA et al., 1991, GOTO et al., 1993).

Oxidative stress under higher oxygen concentration is correlated with the generation of ROS, such as hydrogen peroxide (GOTO et al., 1992) that induces DNA damage in cells (RHAESE; FREESE, 1968, TAKEUCHI et al., 1996).

2.2. Impact of ROS on IVEP and the role of antioxidants

As previously mentioned, the generation of ROS has been pointed out as one of the main causes of the low percentages of IVEP in cattle. ROS is suggested to participate in the meiotic arrest in oocytes (DOWNS; MASTROPOLO, 1994, NAKAMURA et al., 2002), and as a factor of damage and/

or delay of embryonic development *in vitro* (LAMIRANDE; GAGNON 1991, ASHOK et al., 2003).

The gradual increase in ROS levels in 2-cells embryos to the final stage of the morula may depend on the metabolic changes suffered by the embryo during the development (DALVIT et al., 2005). Thompson et al. (1996a) reported a sustained increase in oxygen, glucose and pyruvate uptake during *in vitro* embryo development. This improvement in oxidative metabolism of the embryo may be linked to the detected increase in the ROS levels. On the other hand, the decrease in ROS detected in blastocysts may be related to the beginning of cell differentiation. Thompson et al. (1996b) demonstrated that oxygen consumption decreases in expanded blastocysts because they have lower physiological demand for adenosine triphosphate (ATP), once glycolysis begins to contribute to the production of ATP (THOMPSON et al., 1996a, 2000).

ROS are associated with oxidative stress and cause lipid peroxidation (LPO), protein-incorrect formation, enzymatic inactivation, nuclear DNA tape breaks, mitochondrial damage and expression of apoptotic factors or proteins associated with cell cycle arrest; these phenomena result in blockage of embryonic development and death (HASHIMOTO et al., 2000, GUERIN; EL MOUATASSIM; MENEZO,2001). Besides that, ROS can affect epigenetic marks, including DNA methylation, histone modification, and chromatin remodeling (CHASON et al., 2011).

Therefore, the results of several experiments showed that an antioxidant supplementation in IVEP IVM culture media, to counteract ROS effects, can attenuate the decline in developmental competence induced by oxidative stress (TAKAHASHI et al., 2002; PAPIS et al., 2007; SAKATANI et al., 2007).

An antioxidant is a scavenging enzyme or molecule that converts ROS. Antioxidants are classified as enzymatic or non-enzymatic. Enzymatic antioxidants include superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase. Non-enzymatic antioxidants are also known as synthetic or dietary antioxidants (e.g., vitamin C, vitamin E, β-carotene, selenium, zinc, taurine, and glutathione; AGARWAL, GUPTA, SHARMA, 2005).

2.3. Astaxanthin as an antioxidant in the culture medium

The astaxanthin (AST; 3,30-dihydroxy-b-carotene-4,4'-diona), synthesized by the microalgae *Haematococcus pluvialis* (KOBAYASHI et al., 1997), is a liposoluble antioxidant also found in the red shells of crustaceans, salmon and sea stars (MIKI; YAMAGUCHI; KONOSU, 1982), is a photoprotective red pigment belonging to the carotenoid family and shares that of a similar structure band beta-carotene (TERAO, 1989). It has a wide variety of biological activities, including antioxidant properties (ESTERBAUERET et al., 1987), antitumor (TANAKA et al., 1995) and anti-*Helicobacter pylori* properties (WANG; WILLÉN, WADSTRÖM, 2000).

The AST is a carotenoid with a unique molecular structure containing conjugated double bonds, and hydroxyl (–OH) and keto (C=O) groups, which contribute to its high antioxidant properties (HIGUERA-CIAPARA et al., 2006). AST spans itself across the cell membrane by using its polar end groups to overlap the polar boundary zones of the membrane. In this way, it scavenges free radicals both within the membrane's hydrophobic interior and along its hydrophilic boundaries (GOTO et al., 2001).

It acts as a powerful interceptor of peroxyl (ROO) and alkoxyl (RO) radicals produced during lipid membrane peroxidation (PANASENKO et al., 2000). It is important to note that AST is twice as effective as β-carotene as a peroxyl radical eliminator (GOTO et al., 2001). It has 10 times more active free radical scavengers than other carotenoids (zeaxanthin, lutein, canthaxanthin, β-carotene) and 100 times more than α-tocopherol (NAGUIB, 2000).

Carotenoids are typical antioxidants that eliminate ROS and block LPO, and that AST is superior to β-carotene and vitamin E in the physical extinction of oxygen (DI MASCIO et al., 1990, TATSUZAWA et al., 2000). Also reduces oxidative damage caused by singlet oxygen (DI MASCIO et al., 1989, GOTO et al., 2001, STAHL; SIES 2005) and efficiently eliminates the superoxide anion (CARDOUNEL et al., 2003, JACKSON et al., 2004) and hydroxyl radical (HAMA et al., 2012). In addition, it has effects on some parameters of semen and male fertility (COMHAIRE et al., 2005). AST is also used for the treatment of improved spermatozoid quality (DONÀ et al., 2013). Astaxanthin is also known to have antioxidant properties in the culture of chicken embryo fibroblasts (LAWLOR;

O'BRIEN, 1995). Zenteno-Savin, Clayton-Hernández and Elsner (2002) explained that feeding newborn lambs on a milk substitute enhanced with astaxanthin (6 mg/day) led to an improvement in the quality of meat. However, a limited number of studies on the potential of astaxanthin in ruminants were reported (RAZA et al., 2021).

Li et al. (2015) verified an epigenetic improvement in the maturation of bovine oocytes and embryonic development using AST. Comhaire et al. (2005) reported that astaxanthin inhibits the production of radicals and has a potent antioxidant property. This study also agrees with Kim and Kwon (1999) and Liu et al. (1998) who reported that the antioxidant supplement inhibited LPO in the cell *in vitro* and improved the development of bovine embryos, possibly releasing embryotrophic factors that remove ROS and embryotoxic substances during embryo culture.

Many studies have shown that astaxanthin in *in vitro* culture of mammalian cells has a property of ROS elimination and activation of the cell defense mechanism (CHEN; CHUANG, 1999, BORLOGAN et al., 2000). Jeon et al. (2007) found that astaxanthin improved development rates for the blastocyst stage when swine embryos were grown in PZM-3 medium (medium defined for swine embryos) containing low concentration of astaxanthin (1 ~ 5 μM). The antioxidant effect of astaxanthin was similar to that of different groups (taurine, melatonin, esculetin), which have high antioxidant effect on the development of embryos *in vitro*. Another study found that supplementation of AST to the maturation medium improves the maturation, fertilization and embryonic development of pig oocytes exposed to thermal stress during maturation culture (GUERIN; EL MOUATASSIM; MENEZO,2001), but not the total number of cells.

2.4. Cryopreservation of embryos cultured *in vitro*

Cryopreservation allows the storage of embryos for indefinite periods, contributing to the planning of ovulation and synchronization of both, donors and recipients. In addition, embryonic cryopreservation helps to form a germplasm bank and preserve genetic variability, facilitates the exchange of genetic resources of different breeds and species of economic potential, minimizing the risks of pathogens (WHITTINGHAM, 1971, WILMUT, 1972).

In the last 20 years, numerous modifications have been made to the embryo cryopreservation process in an attempt to improve survival rates and simplify procedures. Vitrification is a simple method for cryopreservation of mammalian embryos, described as a physical process that solidifies a solution at low temperatures, not by crystallization, but by extreme viscosity elevation during cooling (RALL; FAHY, 1985). According to Woods et al. (2004) to obtain this amorphous state, a greater concentration of cryoprotective agents (4 to 8 M) is necessary for traditional freezing (1 to 2 M). These high concentrations prevent the formation of ice crystals. However, this probably causes damage to cells due to osmotic and cytotoxic lesions (KASAI; MUKAIDA, 2004).

Vitrification of bovine embryos produced *in vitro* can be successful with the use of several cryoprotectors: glycerol (GL) and propanediol (PDO) (KUWAYAMA; FUJIKAWA.; NAGAI, 1994); ethylene glycol (EG), ficoll and sucrose (TACHIKAWA et al., 1993, MAHMOUDZADEH et al., 1995); EG and dimethyl sulfoxide (DMSO) (ISHIMORI, et al., 1993, VAJTA et al., 1996); GL and EG (AGCA, 1994, DONNAY et al., 1996). The most common glazing solutions contain an equimolar combination of EG and DMSO, usually 18% or 17% of each cryoprotector (BERTHELOT et al., 2000, CUELLO et al., 2004).

The main lesions are intracellular crystal formations and solute concentration. In the slow freezing of embryos, the ice crystal that forms intracellularly is the main cause of injury (WHITTINGHAM; LEIBO; MAZUR, 1972), while in vitrification, the effect of chemical toxicity of a high cryoprotector concentration is a major obstacle (RALL; FAHY, 1985).

The introduction of a cryoprotector into the cell can cause another type of lesion, osmotic edema. During the cryoprotector removal process, water penetrates faster than cryoprotector, and the embryo degenerates. To avoid this, non-permeable sugar (usually sucrose) is widely used for cryoprotector dilution (LEIBO, 1983, KASAI et al., 1990). However, the hypertonic solution of sugar can cause damage by osmotic retraction (PEDRO et al., 1997).

To minimize these harmful effects, many strategies were developed, based on the use of cryoprotectors of lower toxicity and establishing the ideal volume of the sample, different concentrations, temperature and exposure time (LIEBERMANN et al., 2003), embryo characteristics, such as stage of

development and membrane permeability (ANDREWS; FAHY; FOYER, 2004, BERTOLINI et al., 2005).

The low rate of vitrified-embryonic development is attributed to the process of cryopreservation, which may promote ROS accumulation (AHN et al., 2002, SOMFAI et al., 2007). This evidence indicates that a jeopardized embryonic development associated with vitrification can be, at least, partially preserved by additionally exogenous-supplemented antioxidants.

2.5. Fresh and post-thawing embryonic quality assessments

2.5.1. Evaluation of embryo re-expansion

The ability of embryos produced *in vitro* to survive cryopreservation depends on their quality, which can be affected by cell culture conditions. Thus, cryopreservation tolerance is used as an indicator of embryo quality (PEREIRA et al., 2005).

The post-heating viability of embryos can be evaluated by several methods, but the most common is *in vitro* survival after a period of cultivation (RIZOS et al., 2001).

Grading systems differ with regards to selection of embryo stage and criteria for assessment of embryo quality. There are several stages for the evaluation of preimplantation embryo's quality (NASIRI; EFTEKHARI-YAZDI, 2015).

The code for stage of development is numeric, ranging from "1", an unfertilized oocyte or a 1-cell embryo to "9", expanding hatched blastocyst. IETS Manual's standards for the stages likely to be encountered at that time are described: Morula (Stage code 3); Compact morula (Stage code 4); Early blastocyst (Stage code 5); Blastocyst (Stage code 6); Expanded blastocyst (Stage Code 7); Hatched blastocyst (Stage code 8) (BÓ; MAPLETOFT, 2018).

2.5.2. Nucleus counting and apoptosis index

Hoechst 33342 is a bis-benzimide derivative that binds to AT-rich sequences in the minor grove of double-stranded DNA. The emission is very bright, it is detected at two emission wavelengths after ultra violet (UV) excitation: Hoechst Red (630–650 nM) and Hoechst Blue (405–450 nM). Hoechst 33342

can also be utilized as a DNA binding dye to determine cell cycle status and apoptosis assays, as well as a nucleus indicator in microscopic studies (RAMOS et al., 2003).

Embryos with a higher number of viable cells have greater effectiveness and are expected to maintain potential to establish pregnancies (FERREIRA-SILVA et al., 2017). Similar findings have been described with caprine embryos produced *in vivo,* but data on embryo viability after embryo transfer have not been described (ARAÚJO–LEMOS et al., 2015). According to Byrne et al. (1999), blastocysts that have a small number of cells have a high and variable incidence of apoptosis and the extent of apoptosis is affected by *in vitro* culture conditions. However, the embryonic evaluation with the total number of cells is not faithful in the embryonic viability assessment, and this aspect is best interpreted in conjunction with the apoptosis index.

The TUNEL test is based on the incorporation of triphosphate deoxy uridine nucleotides (dUTP), fluorescently labeled with fluorescein isocyanate (FITC), at the 3'-OH extremities of the fragmented DNA chains, in an enzymatic reaction mediated by the terminal deoxynucleotidyl transferase (TdT) enzyme (GAVRIELI; SHERMAN; BEN-SASSON, 1992). The embedded nucleotides emit a signal, which can then be noticed by flow cytometry or fluorescence microscopy. In the second case, the absence of fluorescence indicates that the TUNEL marking is negative, i.e., that the DNA of the embryo is not fragmented; whereas the green fluorescence emission is indicative of TUNEL-positive marking, that is, that the DNA of the embryo is fragmented. The greater the DNA fragmentation, the more intense the fluorescence emitted. The main advantages of this technique are associated with the fact that it allows simultaneously detecting single chain cuts and double chain DNA, and does not require expensive equipment. On the other hand, this is a quantitative and non-qualitative technique, as it reveals the number of cells with damaged DNA, but not the degree of deterioration of it; there are also some protocol variations between laboratories (SHAMSI; KUMAR; DADA, 2008; ZINI; SIGMAN, 2009).

The number of cells and/ or apoptotic cells in embryos are the common parameters used to evaluate their quality; the addition of antioxidants has been reported to increase the total number of cells and/or reduce apoptotic cells in embryos (MALLOL; SANTALÓ; IBÁÑEZ, 2015; WU et al., 2011).

In the case of the TUNEL test, the apoptotic cells areas when they exhibit TUNEL marking and apoptotic morphology, the criteria for apoptotic morphology are any of the following (GJØRRET et al., 2003): (1) marginal chromatin (i.e., condensed chromatin aggregated in granular masses around the nuclear membrane giving the nucleus an increasing appearance, half-moon or lobulate); (2) pycnotic appearance (i.e., chromatin condensed into a single dense mass, homogeneously colored with roughly spherical or oval shape); (3) multiple fragments consisting of condensed chromatin with varying volumes of multiple densely colored masses; or (4) apoptotic bodies, i.e., multiple fragments scattered in intercellular spaces, eventually producing membrane-bound structures. These cores are usually TUNEL positive. Clusters of nuclear fragments confined in an area comparable to or less than the volume of a normal nucleus are considered to originate from a single nucleus and therefore counted as such. Apoptotic bodies isolated within intercellular spaces are considered to originate from a single nucleus, then dispersed; thus, two to five isolated apoptotic bodies are counted as antiapoptotic nucleus. Nuclei with unclear or thick and swollen edges, or fragmentation into several thick elements with edema are considered necrotic. The proportions of apoptotic and necrotic cells in relation to total cells are called apoptotic index (AI) and necrotic index (IN) (GÓMEZ et al., 2009a, 2009b).

2.5.3. Lipid peroxidation analysis

LPO is a complex process by which polyunsaturated fatty acids in phospholipids are reacted with oxygen to produce lipid hydroperoxide (CHASON et al., 2011). These are reactive carbonyl compounds, largely composed of malondialdehyde (MDA). Therefore, MDA measurement is widely employed as an indicator of LPO (ESTERBAUER; SCHAUR; ZOLLNER, 1991). MDA is composed of fatty acids containing 3 or more double bonds such as linoleic acid and arachidonic acid, and it can be measured via thiobarbituric acid (TBARs) (AKDOGAN et al., 2022).

In LPO, oxidative degradation of various lipids with unsaturated fatty acids, are particularly susceptible to the action of free radicals. LPO damages biological membranes and is therefore highly harmful to their structure and function (DUMOULIN et al., 1999). ROS induce LPO with related effects on cell division,

metabolite transport and mitochondrial dysfunction; in addition, it induces breaks in the nuclear DNA tape that is involved in the stop of embryo development (LOPES et al., 1998). LPO leads to loss of membrane integrity causing increased cellular permeability to electrolytes (HAILLIWELL, 2000). The LPO of the membrane in the cell and the toxicity of peroxides of fatty acids generated are important causes of decreased cell function (GRIVEAU et al., 1995).

It is well known that LPO is increased by high temperature (ALTAN et al. 2003, KANANI et al. 2017), and the AST can counteract this effect (KARPPI et al. 2007, XU et al. 2017). As a highly lipophilic antioxidant, AST is known to prevent the chain reaction of LPO. For example, treatment with astaxanthin reduced LPO in mouse mitochondria both *in vivo* and *in vitro* (BAYIR et al. 2006).

Li et al. (2015) measured the level of LPO and found that levels of 8-iso-prostaglandin F2a, a marker of oxidative stress, decreased significantly after supplementation of AST.

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CHAPTER 2 - THE USE OF ASTAXANTHIN IN *IN VITRO* CULTURE AND SUBSEQUENT EFFECTS ON CRYOPRESERVATION OF BOVINE EMBRYOS

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THE USE OF ASTAXANTHIN IN *IN VITRO* CULTURE AND SUBSEQUENT EFFECTS ON CRYOPRESERVATION OF BOVINE EMBRYOS

ABSTRACT –The objective of this work is to evaluate whether the addition of astaxanthin (AST) to the *in vitro* culture medium (IVC) can exert positive effects on bovine embryos after vitrification. The AST is a carotenoid with high antioxidant properties, due a unique molecular structure containing conjugated double bonds, and hydroxyl (–OH) and keto (C=O) groups, which contribute to eliminate ROS and block lipid peroxidation (LPO). Presumable zygotes were assigned 22 hours after IVF in one of the following treatments: G-CONT) Control, G-DMSO) dimethyl sulfoxide (1 µl DMSO/999 µl IVC medium), G-AST 0.8) 0.8 nM of AST, G-AST 3) 3 nM of AST and G-AST 13) 13 nM of AST. Only grade I expanded blastocysts (XB) on 7th day of development were vitrified (n = 92, 95, 86, 99, and 95/group, respectively). Embryos were evaluated fresh and vitrified for hatching rate and re-expansion, also cell viability including the total number of cells and apoptosis by TUNEL test, in addition to peroxidation lipid levels of culture media with thiobarbituric acid reactive substances (TBARS). Our results showed that there was no difference due to AST supplementation in blastocyst formation on D7. However, the addition of 0.8, 3 and 13 nM of AST into the IVC medium increased the expansion rate of blastocysts after 24 h in the groups G-AST 0.8 (100^{A}(100-100)); G-AST 3 (98^{A}(100-100)); G-AST 13 (100^{A}(100-100)) when compared to G-CONT (93^{B}(100-100)) and G-DMSO (94.3^{B}(100-100)) and 48 h of culture G-AST 0.8 (100^{A}(100-100)); G-AST 13 (100^{A}(100-100)) when compared to G-CONT (91.3^{B}(83.3-100)); G-DMSO (92.4^{B}(80-100)); G-AST 3 (95.5^{AB}(100-100)), independent if the embryo was fresh or vitrified. The apoptotic cell percentage in the embryos after vitrification/warming was higher in the G-AST 0.8 (17.9^{A} (13.5-21.4); ($p<0.05$) compared to G-DMSO 9.7^{B}(8.1-10.7) and G-AST 3 (11.1^{B} (10.0-12.8); ($p<0.05$). TBARS showed no effect of astaxanthin on IVC medium LPO, no differences among the groups to the embryos of the G-CONT at the 7th day of developmental stage ($p > 0.05$). In conclusion, supplementation with AST to the culture medium partially controlled cryoinjury

effects on the vitrified embryos, leading to formation of blastocysts with a comparatively increased rate of XB embryos after warming procedures. Taken together, this study provides new perspectives in understanding how AST could improve the developmental competence of vitrified bovine embryos.

Keywords: astaxanthin, carotenoids, cryoprotectants, reactive oxygen species.

1. INTRODUCTION

Embryos grown *in vitro* may be exposed to oxidative stress for which their defense mechanisms are insufficient to protect their delicate cellular structures, especially those cultured under high oxygen tension (20%). The harmful effects of reactive oxygen species (ROS) during *in vitro* culture (IVC) have been demonstrated in several species. It has been reported that ROS induce mitochondrial dysfunction, DNA, RNA and protein damage [1], as well as in still sperm-oocyte fusion [2]. It seems that the balance between the production and elimination of ROS is an important factor for the acquisition of *in vitro* fertilization (IVF) capacity [3].

To protect oocytes and embryos from oxidative stress during IVC, antioxidants can be added to culture media. Using defined media for all three stages (*in vitro* maturation (IVM), IVF and IVC) and using extracellular enzymatic antioxidants, would allow to know when and what free radical protection is required [4]. *In vitro* produced embryos are held in culture medium for a longer period than in either maturation, or fertilization. As a result, IVC medium is more likely to have a greater effect on the quality of cultured embryos, especially on the timing of development, blastocyst quality, hatching rate, and total cell numbers [5].

Astaxanthin (AST) consists of a special molecular structure on each ionone ring, i.e. presence of hydroxyl and keto molecules, which are accountable for the potent antioxidant properties and superior position in the membrane [6]. AST comprises conjugated double bonds, hydroxyl and keto groups, it has both lipophilic and hydrophilic properties [7] with two oxygenated groups on each ring structure that are responsible for its increased antioxidant characteristics [8]. The AST antioxidant activity has been demonstrated *in vivo* [9, 10] and *in vitro* [11]. The unique structure of AST enables it to extend throughout cell membrane and provides the cell membrane stability. Astaxanthin’s polyene chain traps radicals in the cell membrane, while the AST terminal ring can scavenge radicals on the inner and outer parts of the cell membrane [12]. It also has been shown that AST

has a protective effect on porcine oocytes [13,14] submitted to heat shock. However, the mechanisms by which astaxanthin protects these cells has not been established.

Bovine IVF-derived embryos, after vitrification, usually become extremely vulnerable to cryodamage due to intracellular and membrane defects caused by exchange of water and cryoprotectant agents between the intracellular and extracellular environment [15,16], and the processes of cooling and thawing, which result in osmotic stress. It is believed that an embryo acquires an inherent structural fragility after the cryopreservation process, because it becomes particularly sensitive to the oxidative stress caused by ROS [17,18,19].

Therefore, the present study was conducted to determine whether antioxidant AST at different concentrations added during IVC may be beneficial to blastocyst development, mitigating the effects of cryoinjury in the vitrification process.

2. MATERIALS AND METHODS

All experimental procedures were approved by Committee of Ethics for the Use of Animals (n° 5499/20). All reagents were purchased from SIGMA (Brazil) unless otherwise stated. General procedures for *in vitro* embryo production have been described following the criteria described by Le Guienne [20]. The following are descriptions in brief.

2.1. Oocyte collection and *in vitro* embryo production (IVEP)

Ovaries were collected from slaughtered cows (Frigo West, São José do Rio Preto-SP, Brazil; 20º47'26,635"S 49º23'16,984"W), transported to the laboratory in saline solution and kept at 27 °C - 35 °C during collection and transportation. Cumulus-oocyte complexes (COC) were recovered from 2 to 6 mm follicles with an 18 G needle attached to a 10 mL syringe and deposited in a 50 mL Falcon tube (Falcon®). From this aspirated volume, the supernatant was removed and the pellet was used for the recovery and classification of oocytes, in washing medium containing 25 Mm HEPES (TCM 199 Hepes, Gibco®, Brazil), supplemented with 10% fetal calf serum (FCS Gibco®), 50 µg/mL ciprofloxacin, 2.2 µg/mL pyruvate. Groups of maximum 30 viable oocytes were classified according to the cumulus morphology and cytoplasmic aspect (GI, GII and GIII). After washing, they were matured in 80 µL drops of maturation medium (TCM

199 Bicarbonate medium, $NaHCO_3$ (2.2 mg/mL) supplemented with 10% FCS, 100 IU/mL of hCG, 0.5 µg/mL of FSH, 1mg/mL of estradiol, 2.2 µg/mL of pyruvate and 50 µg/mL of ciprofloxacin), covered with paraffin oil, and incubated at 38.7 °C in 5% CO_2 and high humidity for 22-24h.

For *in vitro* fertilization (IVF; Day 0), commercial frozen semen with proven fertility was thawed and submitted to sperm selection by Percoll gradient (90 and 45%), at 1500g for 5 min in an Eppendorf Centrifuge (Mod. 5418). The pellet was removed and transferred to a second centrifugation containing TALP-IVF medium (modified Tyrode's bicarbonate buffered solution supplemented with penicillamine, hypotaurine, and epinephrine solutions, 10 µg/mL heparin, 6 mg/mL albumin-free fatty acids (FAF)-BSA and 50 µg/mL of ciprofloxacin) for another 3 min. The pellet was recovered, evaluated for sperm motility and concentration and the insemination dose was adjusted to 1 x 10^6 cells/ mL of TALP-IVF. The matured oocytes (maximum 30) were washed in two drops of 100 µL, first in TALP-SPERM medium (Tyrode's albumin lactate pyruvate) and then in TALP-IVF and co-incubated with sperm cells into 50 µL drops of TALP-IVF medium, covered with mineral oil, in the same incubator conditions of IVM during 18 to 20 hours.

The presumptive zygotes were partially stripped, and groups of maximum 30 were assigned in one of the five treatments: G-CONT (negative control - only *in vitro* culture - IVC - medium, composed by modified CR2 supplemented with 3 mg/mL BSA and 5% FCS, In Vitrogen, 16000-036, BME amino acids, MEM non-essential amino acids, 0.1 mg/ mL citrate , 0.5 mg/mL myo-inositol), G-DMSO (vehicle control, 1 µl DMSO/999 µl IVC medium), G-AST 0.8 (IVC + 0.8 nM of AST), G-AST 3 (IVC + 3 nM of AST) and G-AST 13 (IVC + 13 nM of AST). The 80 µL drops of G-AST 0.8, 3 and 13 were covered with mineral oil balanced at the same proposed concentrations of AST. IVC was carried out at 38.7 °C, 5% CO_2 in air with an atmosphere with high humidity. The cleavage rate of the embryos was analyzed on the 4^{th} day post-culture at the time of "feeding" (removing 40 µL and adding another 40 µL of fresh culture medium) and prediction on the 6^{th} day of embryonic development. On the 7^{th} day after fertilization, the embryos were morphologically evaluated and classified according to the International Embryo Technology Society (IETS) standards [21].

2.2. Embryo cryopreservation

2.2.1. Vitrification

Only expanded blastocysts grade I were used in this experiment. Procedures were performed on a heated surface (39 °C). Embryos were immersed into the first drop of vitrification solution 1 (VS1: TCM 199 Hepes + 20% FCS (v/v) + 7.5% EG + 7.5% DMSO) for five minutes. Immediately afterwards the embryos were transferred to the vitrification solution 2 (VS2: TCM 199 Hepes + 20% FCS (v/v) + 15% EG + 15% DMSO + 0.5M sucrose) for one minute. In addition, groups of one to six blastocysts were aspirated into 2 µL of VS2, then they were immediately allocated to the edge of the vitrification device (Vitrific®, WTA, Cravinhos, Brazil) and placed into liquid nitrogen (N_2 L).

2.2.2. Warming

The vitrified embryos were warmed at room temperature for 3s, immersed into the first drop of solution and rehydrated in sucrose of decreasing concentration: step 1) TCM 199 Hepes solution 20% FCS (v/v) + 0.5 M sucrose for 1 min; step 2) TCM 199 Hepes solution 20% FCS (v/v) + 0.3 M sucrose for 3 min; step 3) TCM 199 Hepes solution 20% FCS (v/v) + 0.1 M sucrose for 5 min. Procedures were performed on a heated surface (37 °C).

2.3. Embryo quality analysis

2.3.1. Fresh embryos

2.3.1.1. Expansion and hatching rates

Embryos were maintained in culture to assess expansion, hatching, and survival rate at 24h (D8) and 48h (D9). XB24: percentage of blastocyst on day 8; HB24: percentage of hatching and hatched blastocyst on day 8; SR24: percentage of survival rate within 24 hours; XB 48: percentage of blastocyst on day 9; HB 48: percentage of hatching and hatched blastocyst on day 9; SR48: percentage of survival rate within 48 hours.

2.3.1.2. Apoptosis

Embryos were evaluated by simultaneous assessment of apoptotic index (TUNEL staining) and total nucleus counting. Expanded D7 blastocysts were fixed in 4% (w/v) paraformaldehyde with 0.1 mg/mL of polyvinylpyrrolidone (PVA)

and stored at 4 °C until use. Then, the blastocysts were permeabilized with Dulbecco′s Phosphate Buffered Saline (DPBS) containing sodium citrate (0.1 M; pH 6.0), PVA (0.1 mg/mL) and Triton X-100 (1% v/v) for 1 hour at 4 °C. After permeabilization, samples were then submitted to TUNEL reaction according to the manufacturer's instructions (In situ Cell Death Detection Kit with Fluorescein, 11684795910, Roche®, Mannheim, BW, Germany). For positive controls, samples were treated with 50 IU/mL DNase for 1 hour at 37°C, and then incubated in 10 µL droplets of dUTP nucleotide reaction mixture (TUNEL) mediated by the enzyme terminal deoxynucleotidyl transferase (TdT) under high humidity in the dark. Negative controls were incubated in TUNEL mixture without transferase. After the TUNEL reaction, the nuclei were stained for 15 minutes with 10 µg/mL bisbenzimide in DPBS-PVA. The blastocysts were then washed in DPBS with 0.1 mg/mL PVA and mounted on a glass slide with droplets of 6 µL of 6-diamidino-2-phenylindole (Vectashield-H1000, Vector Labs, USA) under a coverslip [22]. Stained embryos were examined under an epifluorescence microscope (Olympus BX53, Tokyo, Japan) with excitation 330-385nm and emission 420-490nm. DNA-fragmented nuclei were analyzed using software Image Pro Plus (version 7.0). Nuclei with green fluorescence (FITC) were considered TUNEL positive (fragmented DNA). Total healthy nuclei were distinguished from TUNEL positive-apoptotic (APOPTOSIS; % APOPTOSIS) and TUNEL positive-pycnotic (HF) cells by Hoescht staining based on the presence of only blue fluorescence [23].

2.3.1.3. Lipidic peroxidation in culture medium

The IVC media of all replicates were collected, and thiobarbituric acid reactive substances (TBARS) was measured according to adapted methodology described by Ohkawa et al. [24].

Briefly, aliquots of 150 µL of IVC medium and 300 µL of 10% trichloroacetic acid solution (TCA 10%) were centrifuged for 15 minutes (20.817xg) with a temperature of 5 °C. Aliquots of 400 µL of the supernatant were placed in test tubes with 400 µL of thiobarbituric acid at 1% (TBA 1%) dissolved in sodium hydroxide (NaOH) 0.05N, prepared moments before use. The tubes containing this mixture were incubated in a boiling bath (100 ºC) for 15 minutes and cooled in an ice bath (5 ºC) to stop reaction. TBARS were quantified in Ultrospec 3300

Pro Amersham Pharmacia Biosciences®, USA at a wavelength of 532nm. The results were compared with a standard line, previously made with malondialdehyde (MDA; Acros®). MDA is one of the main substances that react with TBA and the concentration of TBARS, lipid peroxidation (LPO) in the medium were expressed in TBARS nanograms/mL of medium.

2.3.2. Vitrified embryos

2.3.2.1. Post-thaw embryo viability

From step 3 of warming, the embryos were cultured in 80 µL drops of Modified CR2 medium covered with mineral oil, incubated at 38.7 °C, 5% CO_2 in air with high humidity. Post-thaw embryo viability was evaluated in terms of re-expansion and hatching rates at 24 h and 48h of culture. Were considered the total percentage of survived embryos (XB24: percentage of blastocyst on day 8; HB24: percentage of hatching and hatched blastocyst on day 8; SR24: percentage of survival rate within 24 hours; XB48: percentage of blastocyst on day 9; HB48: percentage of hatching and hatched blastocyst on day 9; SR48: percentage of survival rate within 48 hours.

2.3.2.2. Apoptosis

The methodology was the same described in 2.3.1.2.

2.3.3. Statistical analysis

The data obtained were analyzed in the SAS System for Windows program (SAS Institute Inc., NC, USA) and tested for normality of residuals and homogeneity of variances. Variables that did not follow these assumptions were transformed (log and square root). The effects included in the model were treatments, embryo (fresh and vitrified), and interaction, which were tested by analysis of variance (PROC GLM), followed by Student's t test. For the comparison between fresh and vitrified, Student's t test and Wilcoxon test were used for parametric and non-parametric variables, respectively. For parametric variables, descriptive data was shown as mean ± SEM; for non-parametric variables, data was shown as median (lower – upper quartile). The significance level used to reject H0 (null hypothesis) was 5%.

3. RESULTS

3.1. Embryo Development

A total of 5060 oocytes produced 1.853 embryos in 13 replicates. Results regarding embryo development until D7 are shown in Table 1. None variable differed between groups.

Table 1. Number and percentages (mean ± SE) of oocyte collection, cleavage, grade 1 and 2 blastocysts evaluated on Day 7, treated with different astaxanthin concentrations, over thirteen replicates.

	Control (n=1007)	**DMSO** (n=1006)	**0.8 nM** (n=1021)	**3 nM** (n=1004)	**13 nM** (n=1022)
Oocytes	77.5 ± 7.1	77.4 ± 6.6	78.5 ± 5.9	77.2 ± 6.2	78.6 ± 7.1
Cleavage	64.1 ± 6.2	66.1 ± 5.5	63.7 ± 5.2	63.8 ± 4.9	65.4 ± 5.8
Cleavage rate	82.9 ± 2.0	86.1 ± 2.0	80.9 ± 1.9	83.4 ± 2.2	83.9 ± 2.7
Blastocysts	29.6 ± 2.9 (n=385)	28.2 ± 2.5 (n=367)	26.6 ± 2.6 (n=346)	28.7 ± 2.9 (n=373)	29.4 ± 3.3 (n=382)
Blastocysts rate	38.6 ± 2.3	36.8 ± 1.7	33.8 ± 1.6	37.9 ± 3.2	37.3 ± 2.2

3.2. Expansion, hatching, and survival rate after 24 and 48h of culture

No interaction was found between treatment and type of embryos, however, a treatment effect (independent of the type of embryo) was found for survival rate 24 h after culture of embryos. G-AST 0.8 [100.0^{A}±0.0; 100 (100-100)], G-AST 3 [98.0^{A}±1.4; 100 (100-100)] and G-AST 13 [100.0^{A}±0.0;100 (100-100) $p < 0.05$] were higher compared to that in the G-Control and G-DMSO [93.0^{B}±2.8; 100 (100-100) and 94.3^{B}±2.1; 100 (100-100), respectively; Table 2]. In addition, the survival rate 48h after culture was similar in G-AST 0.8 [100.0^{A}±0.0;100 (100-100)] and G-AST 13 [100^{A}±0.0 ; 100 (100-100)]; although G-AST 3 [95.5^{AB}±2.0; 100 (100-100)] did not differ from G-control [91.3^{B}±3.1; 100 (83.3-100.0)] and G-DMSO [92.4^{B}±2.3; 100 (80-100)]. No other variables were affected by the treatments ($p > 0.05$, Table 2).

Table 2. Expansion, hatching, and survival rates (mean ± SE; median, lower and upper quartile) of embryos within 24 and 48 hours of culture, according to astaxanthin concentrations (n= 13 replicates).

	Control	DMSO	0.8nM	3nM	13nM	P-value
XB24	57.1±6.0 55 (33.3-75)	49.6±4.5 50 (33.3-66.7)	50.6±5.9 50 (33.3-66.7)	60.7±5.8 66.7 (50-75)	55.8±6.4 50 (40-75)	≥ 0.57
HB24	37.2±6.4 33.3 (0-60)	44.7±4.5 50 (25-66.7)	50.2±6.1 50 (33.3-66.7)	37.3±5.6 33.3 (25-50)	45.5±6.2 50 (25-60)	≥ 0.41
SR24	93^{B}±2.8 100 (100-100)	94.3^{B}±2.1 100 (100-100)	100^{A}±0.0 100 (100-100)	98^{A}±1.4 100 (100-100)	100^{A}±0.0 100 (100-100)	≤ 0.01
XB48	15.5±4.2 0 (0-33.3)	19.2±5.5 0 (0-33.3)	19.4±5.5 0 (0-33.3)	26.1±6.7 16.7 (0-33.3)	23.0±5.2 8.4 (0-50)	≥ 0.70
HB48	75.8±6.3 100 (66.7-100)	73.1±5.8 75 (60-100)	81.4±5.6 100 (66.7-100)	69.4±6.4 75 (66.7-100)	78.3±5.3 100 (50-100)	≥ 0.78
SR48	91.3^{B}±3.1 100 (83.3-100)	92.4^{B}±2.3 100 (80-100)	100^{A}±0.0 100 (100-100)	95.5^{AB}±2.0 100 (100-100)	100^{A}±0.0 100 (100-100)	≤ 0.01

Note: Values in the same column with different letters are significantly different (p<0.05). **XB24)** Expanded blastocysts rate within 24 hours pos warming. **HB24)** Hatching and hatched blastocyst rate within 24 hours pos warming. **SR24)** Embryos rate within 24hours. **XB48)** Expanded blastocysts rate within 48 hours pos warming. **HB48)** Hatching and hatched blastocyst rate within 48 hours pos warming. **SR48)** Embryos rate within 48hours.

Regarding the type of embryo (independent of treatment), fresh embryos had a greater result on the hatching and survival rate within 24h and 48 hours when compared to vitrified ones. Inversely, expansion rate at 48h was greater for vitrified instead of fresh embryos (Table 3)

Table 3. Expansion, hatching, and survival rates (mean ± SE; median, lower and upper quartile) of embryos cultured for 24 and 48 hours, according to type of embryos – fresh or vitrified (n= 13 replicates).

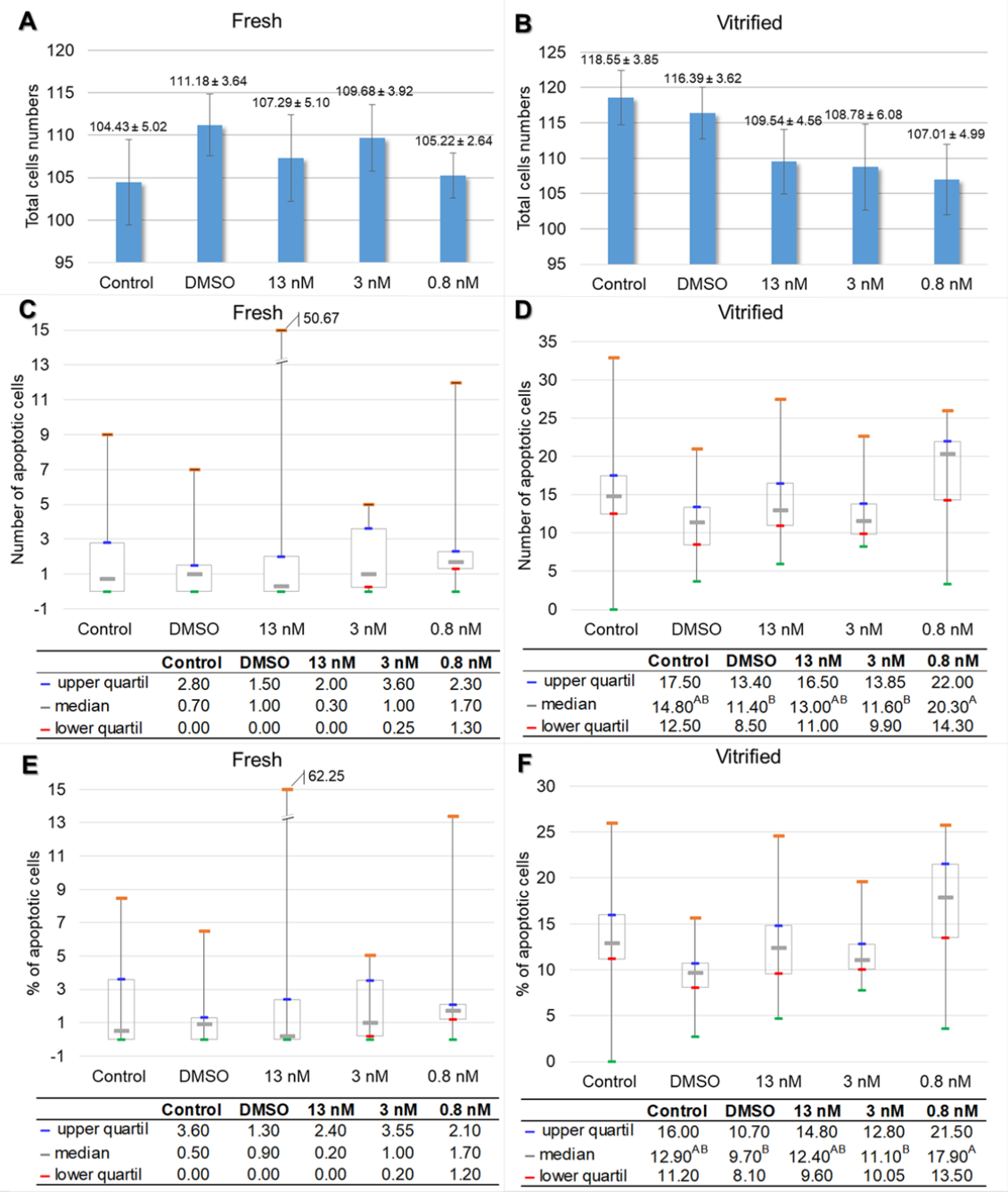
	FRESH	VITRIFIED	P-value
XB24	51.3±3.6 50 (33.3-66.7)	58.1±3.7 60 (33.3-75)	≥ 0.18
HB24	48.3±3.5 50 (33.3-66.7)	37.6±3.8 33.3 (0-63.4)	≥ 0.03
SR24	98.9±0.7 100 (100-100)	95.2±1.4 100 (100-100)	≤ 0.01
XB48	11.7±2.7 0 (0-22.5)	29.6±3.8 33.3 (0-50)	≤ 0.01
HB48	87.0±2.7 100 (75-100)	64.3±4.0 66.7 (50-100)	≤ 0.01
SR48	97.8±0.9 100 (100-100)	93.8±1.6 100 (100-100)	≥ 0.01

XB24) Expanded blastocysts rate within 24 hours pos warming. **HB24)** Hatching and hatched blastocyst rate within 24 hours pos warming. **SR24)** Embryos rate within 24hours. **XB48)** Expanded blastocysts rate within 48 hours pos warming. **HB48)** Hatching and hatched blastocyst rate within 48 hours pos warming. **SR48)** Embryos rate within 48hours.

3.3. Total cell number and apoptotic cell percentage in the blastocysts

The total cell number was not affected by treatment (P = 0.46), type of embryo (P = 0.11) or interaction (p = 0.49; Fig 2 A and B).

The number of apoptotic cells and apoptotic index was not affected by the treatments (p =0.46 and 0.32, respectively), however both were influenced by the type of embryos (P < 0.01 for both variables, Fig 2 C to E).



C	Control	DMSO	13 nM	3 nM	0.8 nM
upper quartil	2.80	1.50	2.00	3.60	2.30
median	0.70	1.00	0.30	1.00	1.70
lower quartil	0.00	0.00	0.00	0.25	1.30

D	Control	DMSO	13 nM	3 nM	0.8 nM
upper quartil	17.50	13.40	16.50	13.85	22.00
median	14.80^{AB}	11.40^{B}	13.00^{AB}	11.60^{B}	20.30^{A}
lower quartil	12.50	8.50	11.00	9.90	14.30

E	Control	DMSO	13 nM	3 nM	0.8 nM
upper quartil	3.60	1.30	2.40	3.55	2.10
median	0.50	0.90	0.20	1.00	1.70
lower quartil	0.00	0.00	0.00	0.20	1.20

F	Control	DMSO	13 nM	3 nM	0.8 nM
upper quartil	16.00	10.70	14.80	12.80	21.50
median	12.90^{AB}	9.70^{B}	12.40^{AB}	11.10^{B}	17.90^{A}
lower quartil	11.20	8.10	9.60	10.05	13.50

Figure 1. Comparison between fresh and vitrified in vitro–produced bovine embryos, according to treatments with AST. A and B: total cell number, C and D: number of apoptotic cells, E and F: % of apoptotic cells.

3.4. Lipid Peroxidation

The content of TBARS in culture medium of all the groups did not differ among the treatments (p = 0.77, Fig. 3).

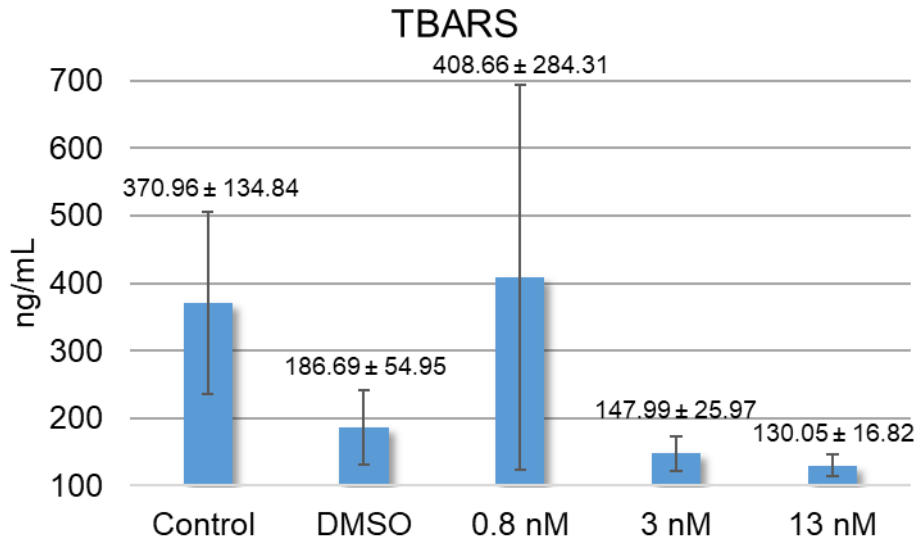


Figure 2. Lipid peroxidation measured by TBARS nanograms/mL) produced during the period of culture under different astaxanthin concentrations (n = 13 replicates).

4. DISCUSSION

In the present study, AST supplementation was performed during embryo *in vitro* culture and their effects evaluated after vitrification for the first time. The addition of antioxidants to IVC medium might be a prerequisite for improvement of developmental competence of embryos by *in vitro* production [25,26], mainly when culture occurs in high O_2 tension. Embryo protection against ROS depends, in part, on an endogenous pool of antioxidant enzymes [27]. Different astaxanthin concentrations were added to the culture medium, however, inversely to expected, the majority of our results on embryo developmental competence were not affected by AST. Other studies have demonstrated the beneficial role of astaxanthin on heat-shocked bovine embryos, when this antioxidant was added during IVM [13,28].

Cryopreservation of embryos has been confirmed to cause oxidative stress as a factor responsible for impaired developmental competence. However, there has been little attention on the effects of antioxidants post-culture, on the cryosurvival of vitrified–warmed embryos [29]. Xiang et al. [30] showed that 1.5 μM of AST supplementation during IVC achieved the highest blastocyst yield for

porcine parthenogenetic zygotes after vitrification. Moreover, this concentration also increased the blastocyst formation rate of vitrified porcine zygotes. The addition of 0.8, 3 and 13 nM of AST into the IVC medium increased the expansion rate of blastocysts after 24 h and 48 h of culture, independent if the embryo was fresh or vitrified; this difference became significant compared with G-Control and G-DMSO. These results were similar from those reported by Salzano et al. [31], who informed an increased developmental rate 24 h following vitrification and warming after a supplementation of 0.5 μM of resveratrol during IVC. Other studies have also reported that resveratrol supplementation during embryo culture or during a 6 or 24 h pretreatment before cryopreservation improves viability and hatching rates after thawing [15,32]. Despite of the results of these other studies, our AST treatments during embryo culture also showed a higher percentage of expansion and hatching rate (collectively) within 48 hours. Surprisingly, the G – AST 3 nM presented the same rate as all other treatments, likewise the embryos presented higher expansion rate within 48 hours. The percentage of hatching blastocysts and the average number of cells/blastocysts depend on the quality of oocytes and the quality of sperm used by each laboratory [33]. Concentrations need to be carefully defined so that only the optimal amount is used to counteract the ROS excess, while maintaining a physiologic level of ROS. This suggests that the balance between the ROS production and the detoxification effect with AST are an important factor in embryo survival. Despite of AST antioxidant property, the optimal concentration of antioxidants is important because it can have negative as well positive effects, depending on the concentration of the supplement in the medium.

In addition, total cell counting and, particularly, cell allocation are used as embryo quality parameters. In the present study, embryos produced in the medium enriched or not with AST showed that the total cell counting were unaffected for fresh and vitrified embryos, which is in accordance with Cuello et al. [34], that obtained similar total cell numbers when compared fresh and vitrified/warmed embryos. However, Dhali et al. [35] obtained a greater number of cells in fresh embryos compared to vitrified/warmed embryos.

As expected, higher TUNEL-positive cell percentages occurred in frozen blastocysts than in their fresh control counterparts, as also demonstrated in studies in human, mouse, and bovine blastocysts [36,37]. Since *in vitro* embryos

production (IVEP) are susceptible to cryopreservation, subtle changes in the cryoprotectant concentration, freezing speed, length of exposure to cryoprotectants, and operator skills may affect the outcomes. It is now evident that both fresh embryo development and vitrification leads to physical, chemical, and thermal damage, which could lead to induction of apoptosis in blastocysts [38].

An imbalance between the formation of ROS and antioxidant capacity [38] can lead to many pathological effects, such as LPO, ATP depletion, mitochondrial alterations, and fetal growth arrest. The difference in the oxidative stress responses between the stages may be explained due to the fact that developing organisms differ in oxidative stress situations and antioxidant strategies during their embryonic development [39]. Astaxanthin's ability to penetrate biological membranes with a defensive effect of LPO within and outside of the cell membrane is one of its main features [40]. It is well known that LPO is enhanced by elevated temperature [41,42] whereas astaxanthin can neutralize this effect [43,44]. In our study the supplementation of the IVC medium with AST did not improved embryo developmental rates, although there was no effect of temperatures.. Moreover, Kumar and Singh [45] has shown decreased levels of stress in heifers and potent antioxidant activity of AST in treatment group after TBARS and LPO. Although a decrease in the TBARS levels (LPO) was not observed in the present study, there is a lack of knowledge regarding oxidative stress caused directly by the culture medium and culturing techniques. It has been reported that ROS can be generated spontaneously by buffers and enriched culture media. Moreover, diverse oxidase activity is present in the serum components and serum replacements, which may speed up and amplify ROS production in these buffers [46]. Therefore, it seems that while developmental competence and quality of embryos did not differ regarding the stage of antioxidant supplementing in this study, much of the effort has been devoted only to the effect of AST in pre-embryo development before cryopreservation, rather than its effect on post-vitrification embryo development. Consequently, the speed of recovery after thawing may depend not only on the integrity of the embryo itself but also on the nature of the post-thawing culture environment.

Furthermore, before using this additive routinely for commercial purposes, more studies are needed to investigate the exact mechanism and beneficial effect by which AST protects the cryopreserved embryo.

5. CONCLUSION

In conclusion, the results of this study taken together demonstrated that enrichment of bovine IVC medium with different AST treatments can partially improve cryotolerance after culture, due the better survival rates within 24 and 48 hours when compared to the control. Therefore, AST as a powerful exogenous antioxidant requires further studies to apply more efficiently in the IVC system for recovery culture of the vitrified bovine embryos or other reproductive biology field.

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Declaration of Competing Interest

The authors declare no conflicts of interest.

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Author contributions

Fernanda Aidar: Conceptualization, Investigation, Methodology, Data curation, Writing and original draft, review & editing. Lindsay Unno Gimenes:

Conceptualization, Methodology, review & editing. Ligia Garcia Mesquita: Methodology. Marcílio Nichi: Methodology and Statistical analysis. Cláudia Maria Heredias Ribas: conceived, performed the experiments and laboratory analyses, analyzed the data and prepared the draft manuscript. All authors critically reviewed the manuscript and approved the final version.

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