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CÂMPUS JABOTICABAL

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

Fernanda Said Aidar
Médica Veterinária

2023

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

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Dissertação de mestrado apresentada à Faculdade de Ciências Agrárias e Veterinárias – Unesp, Campus Jaboticabal, como parte dos requisitos para obtenção do título de Mestre em Medicina Veterinária.

2023

A288u

Aidar, Fernanda Said

Uso de astaxantina em cultivo in vitro de embriões bovinos e efeitos na criopreservação / Fernanda Said Aidar. -- Jaboticabal, 2023

59 p.

Dissertação (mestrado) - Universidade Estadual Paulista (Unesp), Faculdade de Ciências Agrárias e Veterinárias, Jaboticabal

Orientadora: Profa. Dra. Lindsay Unno Gimenes

1. Astaxantina. 2. Carotenoides. 3. Crioprotetores. 4. Espécies reativas de oxigênio. I. Título.

Sistema de geração automática de fichas catalográficas da Unesp. Biblioteca da Faculdade de Ciências Agrárias e Veterinárias, Jaboticabal. Dados fornecidos pelo autor(a).

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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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Aprovada como parte das exigências para obtenção do Título de Mestra em Medicina Veterinária, área: Reprodução Animal pela Comissão Examinadora:

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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.

ACKNOWLEDGMENTS

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.

To my brother Renato (*in memorian*) who taught me that we are not better than anyone else, that we live in a society where we are all equal and that from up there, wherever he is, he always watches over me and guides my steps. I'm sure of that, one day we'll meet again!!!

To my aunts San and Sé, who are my base, my strong axis, the ones who never let the shuttlecock fall, who always did the possible and the impossible to make my life a bed of roses. I love you "Favorite Aunts"

To Prof. Dr. Lindsay Unno Gimenes who bet all her chips that an "old woman" like me could return to the academic world and show that she still does the job. For guiding me, teaching and trying to get the best out of this Fernanda here. Grateful for teaching me how to fish the fish and not put it on the hook!!!!

To Dr. Ligia Garcia Mesquita from the Department of Nutrition and Animal Production of the Faculty of Veterinary Medicine and Animal Science (FMVZ-USP), for putting up with me every day asking the toughest questions that only a Virgo with a fertile imagination like me would do, with all my "…...but what if that happens." The Tunel test became the easiest technique and was far from looking like a seven-headed beast when you joined the team to help. Also, a big thanks to Luisa Pestana and my stepmother Ana Silveira who had an important role in one of the steps of Tunel technique.

To Prof. Dr. Alessandra Vidotto, coordinator responsible for the Multiuser Laboratory of FAMERP - Faculdade de Medicina de São José do Rio Preto (LMU) together with her assistant Andrea Rodrigues Santos who allowed my slides to be read in my city during this time of pandemic, preventing I had to move there.

To Prof. Dr. Marcílio Nichi and his doctoral student Raphaela Gabrielle Brito Sousa from the Animal Reproduction Department of the Faculty of Veterinary Medicine and Animal Science (FMVZ-USP), for having collaborated with the TBARs technique. It would never be possible to replicate outside your

laboratories and help me out with the statistics of my work in the 42 seconds of the second half, so that I could take my research project to the SBTE congress.

To Prof. Felipe Perecin from Department of Veterinary Medicine of the Faculty of Animal Science and Food Engineering (FZEA-USP), and Prof. Dr. Anelise Peres from the Division of Animal Welfare, Secretary of the Environment, Municipality of Ribeirão Preto who helped me with all the dilution accounts so that Astaxanthin reached the desired concentrations. Mathematics was never really my strong point, I think that's why I chose the area of biological rather than the exact science. Grateful for helping me to think together about the way to dilute and save the use of the mediums for the embryo production.

To the Reproduction Area from Department of Pathology, Reproduction and One Health, of the School of Agricultural and Veterinarian Sciences (FCAV), I thank everyone for all the help with the bureaucratic part, but mainly to Branca Rochidali José for having clarified all my doubts regarding the system over these 2 years.

To my partner and best friend Eva, grateful for playing all the weekend routines. And to all the interns who spent time with us during this period to make this happen, each of you has a little bit of this Master's degree.

To Claudia, who was Jaboticabal's greatest gift. The only bad part of our friendship was not living in the same city so we could do anything together. I admire you, friend! Thank you for being so special and present in my life. I will always remember that if I got this far it was because you were around every obstacle I passed.

To Aline, who not only became my Master's partner, but also one of my right-hand men within FertVitro, helping me with the commercial routine so that I could distribute my time between the early hours that an *in vitro* fertilization laboratory take and the studies that the Master's degree requires. You made all the difference in my team. Thanks for everything!!!

To Marina, Alessandra and Joice for being able to share the crazy ideas of "Prof. Pardal". Help to share the fear and uncertainty if this would work, but rest assured, at the end everything works out. If it didn't work out, it's because it didn't make it to the end!

To my friends Willian Galheira and Vanessa Massini who loaded and unloaded boxes and boxes of consumables for our experiments from Ribeirão

Preto/Jaboticabal to São José do Rio Preto so that the routine could take place without the extra cost of mailings. Not even a taxi would do what you did for us. My immense gratitude for all the help!!!

To the WTA® company for providing the cryotops devices and to FertVitro for allowing the experiments to be carried out within a commercial laboratory.

To all the people who helped me during this process, thank you so much!

“O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001.”

“This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001.”

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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
Coordenadora – CEUA

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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.

2.1. 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).

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.

Declaration of funding

This study was supported by Higher Education Personnel Improvement Coordination– Brazil (CAPES, Financial Code 001) and the Programa de Pós-Graduação em Medicina Veterinária da Faculdade de Ciências Agrárias e Veterinárias (FCAV-UNESP).

Declaration of Competing Interest

The authors declare no conflicts of interest.

Ackowledgments: Faculty of Medicine of São José do Rio Preto (FAMERP - Faculdade de Medicina de São José do Rio Preto), FertVitro - Laboratório de Fertilização *In Vitro*, Faculty of Agricultural and Veterinary Sciences (Faculdade de Ciências Agrárias e Veterinárias), WTA – Solutions for Animal Reproduction (Soluções para Reprodução Animal) and Higher Education Personnel Improvement Coordination– Brazil (CAPES, Financial Code 001)

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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