UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS
DEPARTAMENTO DE BIOLOGIA ANIMAL

Effect of mitochondriotropic molecules in reducing oxidative stress in assisted reproduction techniques

Beatriz Lourenço

Mestrado em Biologia Evolutiva e do Desenvolvimento

Dissertação orientada por:
Doutora. Rosa Lino Neto Pereira
Prof. Doutora Gabriela Rodrigues

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Resumo:

A infertilidade está a aumentar, sobretudo por problemas de origem genética ou patológica do casal, mas também devido a fatores externos como nutricionais, estilo de vida e de impacto ambiental. O fator ambiental é notório desde a revolução industrial e em alguns animais já se estuda os transtornos que as alterações climáticas têm na fertilidade. Nos humanos, o estilo de vida e o adiamento da gravidez, são os fatores externos que mais influenciam a infertilidade, com redução da qualidade dos gametas e prejuízo do ambiente uterino e desenvolvimento embrionário.

A reprodução medicamente assistida (RMA) tem vindo a atenuar alguns dos problemas associados à infertilidade desde 1978, data do nascimento do primeiro bebé de fertilização *in vitro (FIV).* No entanto, apesar de grandes evoluções nas técnicas de RMA, a taxa de sucesso continua baixa. Apenas 30% dos casais diagnosticados com algum tipo de infertilidade conseguem levar uma gravidez até ao final.

Tanto em gravidezes naturais como em gravidezes auxiliadas por RMA, um fator que pode influenciar a qualidade dos embriões é o nível de Reactive Oxygen Species (ROS). As ROS ocorrem naturalmente no processo de respiração celular mitocondrial, resultantes da redução das moléculas de oxigénio, e tem um papel importante na resposta celular. No entanto, dado à toxicidade de ROS, os seus níveis são estritamente controlados por antioxidantes endógenos. Quando a resposta endógena falha e os níveis de ROS ficam acima do limite, a célula entra em *stress* oxidativo. Este stress pode ser devido a deficiências nutritivas, e em casos de recurso a RMA, pode ser oriundo de exposição à luz, choques térmicos ou osmóticos, como também ao desenvolvimento embrionário em ambientes ricos em oxigénio. O *stress* oxidativo pode levar à morte celular, à pior qualidade embrionária e em última estância à perda do feto. Neste trabalho é abordada a falta de terapias eficientes para a questão do *stress* oxidativo na reprodução assistida e a intenção da obtenção de um tratamento para o mesmo.

O problema do *stress* oxidativo é algo recorrentemente estudado, sendo usadas diferentes terapias que incluem a suplementação dos meios de cultura com antioxidantes. Estes antioxidantes, tal como os antioxidantes endógenos, têm o papel de eliminar o ROS ou de manter a célula num estado redox, para manter níveis de ROS saudáveis. Muitos antioxidantes utilizados como terapia já foram testados e mais recentemente a adição de antioxidantes com a mitocôndria como alvo obtiveram bons resultados, devido à síntese de ROS ser realizada principalmente na mitocôndria.

Neste estudo, pretendemos testar a inclusão de novos antioxidantes dirigidos para as mitocôndrias no meio de cultura dos embriões, de forma a aumentar a qualidade de embriões viáveis produzidos. Estudámos dois antioxidantes inovadores, o AntiOxBEN2 e o AntiOxCIN4, e investigámos os seus efeitos, qual a melhor concentração dos diferentes fármacos e a comparação entre os dois em embriões posteriormente submetidos ao processo de criopreservação. Para este estudo foi utilizado o bovino como modelo animal, onde os oócitos foram aspirados dos ovários proveniente do matadouro e maturados em laboratório, seguindo-se a fertilização *in vitro* e a cultura dos pressupostos zigotos em meio de cultura suplementado com os antioxidantes produzidos pela nossa equipa. O estudo foi dividido em 3 experiências principais, sendo que as duas primeiras se debruçaram na testagem das melhores concentrações dos dois antioxidantes e, a terceira experiência, sobre a comparação dos dois antioxidantes nas suas melhores concentrações em embriões posteriormente criopreservados. Os 3172 pressupostos zigotos foram divididos nas 2 experiência iniciais sendo que a primeira experiência, testou 3 doses de AntiOxCIN4 (1µM CIN; 2.5 µM CIN; 10 µM CIN) e a segunda experiência testou 3 doses de AntiOxBEN2 (1µM BEN; 2.5 µM BEN;10 µM BEN), sempre comparadas com um grupo

controlo, sem antioxidante. Nos dias 2, 7 e 9, os embriões de cada experiência foram analisados morfologicamente (taxas de desenvolvimento e qualidade). As células totais e viáveis foram examinadas, como também os estados da mitocôndria, através do potencial de membrana e por fim, foram avaliados os níveis de ROS. Todas as analises, excetuando as morfológicas, foram realizadas com corantes fluorescentes específicos. Nestas primeiras experiências, os melhores resultados foram obtidos com as concentrações de 2.5µM CIN e 2.5µM BEN. A dose de 2.5 µM CIN destacou-se pela diminuição de embriões de má qualidade (P= 0.031) e a de 2.5 µM BEN por um maior número de embriões de boa qualidade (P=0.0014) e um acréscimo no número total de células dos embriões (P=0.01). Assim, estas duas concentrações foram selecionadas para a terceira experiência

Na terceira experiência, os pressupostos zigotos forma divididos em três grupos: controlo; 2.5µM CIN; 2.5µM BEN. Posteriormente os embriões com qualidade (grau 1 e 2) foram vitrificados e descongelados. Foram igualmente analisados quanto à morfologia antes e depois da congelação, incluindo as taxas de criosobrevivência (integridade e expansão) e de células totais e viáveis, estado da mitocôndria e níveis de ROS, como nas experiências anteriores. Nesta última experiência, os dois antioxidantes obtiveram bons resultados nos ensaios de qualidade, no entanto AntiOxBEN2 obteve os melhores resultados nas expansões dos embriões após descongelação e cultura (64.7% dos embriões expandiram com a concentração de 2.5BEN enquanto o grupo controlo só obteve 36.4% de embriões expandidos, P=0.05). O Grupo 2.5 CIN também apresentou melhores taxas de expansão quando comparado com o controlo (P=0.09). Resultados anteriores da nossa equipa demonstraram que o AntiOxCIN4 tinha uma maior capacidade antioxidante, mas que o AntiOxBEN2 era mais dirigido à mitocôndria, revelando-se um antioxidante mais estável. O AntiOxBEN2 demonstrou ser uma melhor terapia para o stress oxidativo em embriões, aumentando a sua qualidade e a capacidade de resistir ao processo de criopreservação.

Este estudo teve algumas limitações no número de embriões produzidos. Tendo em conta a quantidade de ensaios que foram realizados, com elevado número de doses a testar devido ao carater inovador da aplicação dos antioxidantes desenvolvidos, seria necessário um N maior para podermos ter algumas conclusões mais robustas nas análises do estado da mitocôndria e nos valores de ROS. Esses dados seriam essenciais para aferirmos sobre como os antioxidantes afetam a mitocôndria e se de fato há uma redução de ROS durante a cultura dos embriões. Para superar essa limitação, analises complementares teriam de ser feitas para haver uma confirmação dos nossos dados. Seria ainda benéfico fazer um estudo sobre como AntiOxCIN4 e AntiOxBEN2 influenciam a expressão genica de alguns componentes essenciais para a recuperação das células após o stress oxidativo. Apesar de já existirem vários estudos a defender que estes antioxidantes tem uma influência positiva no pool de antioxidantes endógenos, esta informação terá de ser confirmada em embriões.

Apesar de ser necessário alguns estudos para a corroboração de dados, AntiOxCIN4 e AntiOxBEN2 já revelaram ter resultados promissores e até mesmo demonstraram ter capacidades para competir com antioxidantes que já se encontram no mercado como por exemplo o MitoQ10. O MitoQ10 apesar de ter uma grande capacidade de antioxidante releva-se toxico para as células até mesmo em baixas concentrações, enquanto o AntiOxCIN4 e o AntiOxBEN2 em concentrações baixas demonstram excelente capacidade antioxidativa e sem toxicidade. A suplementação destes dois antioxidantes, principalmente do AntiOxBEN2, no meio de desenvolvimento dos embriões tem implicações benéficas para o desenvolvimento embrionário, sendo uma promissora terapia para o stress oxidativo e uma ótima solução para as técnicas de RMA.

Abstract:

Over the past decades, an expansion of assisted reproductive techniques (ART) has been reported. Even so, the process continues to have a low success rate, being necessary to optimize these procedures. One of the setbacks of ART is the oxidative stress, impairing the embryo development, namely by delaying this development or even inducing cell death or apoptosis.
In this work, the lack of effective therapies for oxidative stress in ART is approached as well as the aim of getting a reduction of the oxidative stress during embryo production. We hypothesised that the inclusion of new antioxidants directed to mitochondria in the embryo culture medium improves the quality of viable embryos. We have studied two different antioxidants, AntiOxBEN2 and AntiOxCIN4 that were synthesised by our team. This work was performed with cattle gametes in three experiments. Abattoir derived oocytes were matured and submitted to *in vitro* fertilization, and the presumptive zygotes were divided into culture medium supplemented with antioxidants. In the first and second experiments, we have investigated the effects of the antioxidants on embryo development and tested their best concentrations. The first experiment focused on the effects of AntiOxCIN4 at the concentration of 1 μM, 2.5 μM, and 10 μM and the second on the effects of the AntiOxBEN2 at the concentration of 1 μM, 2.5 μM, and 10 μM. A control group without supplementation run simultaneously. The third experiment had the aim to compare the two antioxidants molecules in their best concentrations as well as the performance of cryopreserved embryos. In each experiment, embryos were morphologically evaluated, the total and viable cells were examined, as well as ROS levels and the mitochondrial state, through the membrane potential evaluation, using specific fluorescent dyes.

With this novel therapy, in experiments 1 and 2, we achieved an increase of embryo quality on the concentrations of 2.5 μM AntiOxCIN4 (P= 0.031) and 2.5 μM AntiOxBEN2 P=0.0014). In the experiment 3, an improvement in the cryosurvival in both antioxidants, being the AntiOxBEN2 standing out with the best results (expansion rates after vitrification/warming and culture, P=0.05).

This study had some limitations due to the low number of embryos, which caused some doubts in the mechanism that caused the embryo quality increased, and more studies will be needed to understand this process. However, it was found the concentration that achieved goods results in the embryo's development and in cryosurvival which points to a possible good therapy for oxidative stress.

Keys words: Desenvolvimento embrionário, Stress oxidativo, Reprodução assistida, ROS, antioxidantes dirigidos para a mitocôndria

Palavras chave: Assisted reproduction, Embryonic Development, Oxidative stress, ROS, antioxidants directed to mitochondria

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List of abbreviations:

ART - Assisted Reproductive Technologies

ATP – Adenosine triphosphate

BEN – AntiOxBEN2

BSA - Bovine serum albumin

CAT - Catalase

CIN- AntiOxCIN4

CO_2 – Carbon Dioxide

COC – Cumulus-oocyte complexes

CoQ10 – Coenzyme Q10

D7 – Day 7^{th}

D9 – Day 9^{th}

DNA – Deoxyribonucleic acid

EDTA – Ethylenediamnine tetraacetic acid

EG - Ethylene glycol

EGF - Epidermal Growth factor

ETC - Electron transport chain

FCS - Foetal bovine serum

Fe^{2+} -Ferrous ion

Fe^{3+} - Ferric ion

FERT- Fertilization medium

GPx - Glutathione peroxidase

GST - Glutathione S-transferases

H_20_2 - Hydrogen peroxide

H_2O - Water

HMOX1 – Heme Oxygenase 1

ICSI - Intracytoplasmic sperm injection

INIAV – Instituto NacionalAgrária e Vetrinária

IT - Gamete Intrauterine Transfer

IVF - *In Vitro* Fertilization

JC-1 - 5, 5´, 5´-tetrachloro-1, 1´, 3, 3´- tetraethylbenzimidazolcarbocyanine iodide

LO - Alkoxyl

LOG - Lipid Peroxyl

MitoT- Mito-TEMPO

MMP - Mitochondria Membrane Potential

MtDNA – Mitochondrial DNA

N_2 - Nitrogen

NAD – Nicotinamide adenine dinucleotide

NADH - Nicotinamide adenine dinucleotide phosphate hydrogen

NBSCS - Newborn calf serum

NFR2- Nuclear factor erythroid 2-related factor 2

NQQ1- Quinone oxidoreductase 1

O_2 – Oxigen

$O2^{\cdot}$ - Superoxide

OH^{-} - Hydroxyl anion

OH^{*} - Hydroxyl radical

OXPHOS - Oxidative phosphorylation

PAINS - pan-assay-interference-compound

PBS - Phosphate buffered saline

PCOS - Polycystic ovarian syndrome

PI - Propidium iodide

ROS - Reactive Oxygen Species

SkQ1 - Visomitin

SOD - Superoxide dismutase

SOF - Synthetic oviductal fluid

SNO - S-Nitroso

TCM199 - Tissue Culture Medium

TPP - Triphenylphosphonium

ΔΨm - Mitochondrial Membrane Potential

Introduction:

Infertility is diagnosed when couples are incapable to conceive after a year of unprotected sexual relationships (Zegers-Hochschild et al., 2009; Renzi et al., 2020). This condition affects nearly 48.5 million couples (Mascarenhas et al., 2012; Renzi et al., 2020), distressing one in every six couples worldwide (Sharlip et al., 2002; Martin-Hidalgo et al., 2019). Infertility can be caused either by female issues (40%), male issues (40%), or a combination of both (10%). The remaining 10% are cases of infertility without explanation (Gameiro, 2016; Wagner et al., 2018). The most frequent source of infertility is due to an inferior number and quality of germ cells, namely Azoospermia and Oligospermia for men, or the failure of ovulation caused by hormonal disorders, such as polycystic ovarian syndrome (PCOS) in women. Infertility can also be caused by injuries on the pelvic cavities such as endometriosis, disorders in the Sertoli cells, or obstruction of spermatic ducts (Dominguez & Reijo Pera, 2013; Gameiro, 2016; Szkodziak et al., 2016; Kiesswetter et al., 2020)

Infertility as well as other health issues could be linked to multiple factors, not only of genetic origin and pathological problems but also to nutritional (Gaskins & Chavarro, 2018), environmental, and lifestyle disorders (Luoma, 2005). For example, a healthy diet should promote a lower risk of ovulatory disease in women, while increasing sperm quality in men (Chavarro et al., 2007; Gaskins & Chavarro, 2018), even though some of the components present in this diet like vegetables, grains and fish have hight levels of environmental toxicants (Gaskins & Chavarro, 2018).

The environmental factor plays a important role, mainly after the modern chemical revolution in the last half of the past century. Pesticides, detergents, plastics, and cosmetics were released into the environment, some of them being qualified as reproductive toxicants living in our body, and transported by air, soil, food, and water (Luoma, 2005; National Health and Nutrition Examination Survey (U.S.), 2005). A study by Guillette & Edwards (2005) proved that alligators from a pesticide-contaminated lake suffer from reproductive problems. The same happens with toxic pesticides in mice (Luoma, 2005; Kaiser, 2016) and a relation between human fertility and the environment toxics was studied as well (reviewed in Luoma, 2005).

Like the environment that surrounds us, our lifestyle is likewise an important factor. The consumption of alcohol, coffee, oral contraceptives, and smoking put at risk the women´s/man´s fertility (Chavarro et al., 2007; Panth et al., 2018). Delayed childbearing acts as the major factor that contributes to infertility in this category. The average age of women at her first child is now 30 years old and this age is constantly rising (Guzick & Swan, 2006; Brandt et al., 2019; Somigliana et al., 2020).

By the age of 31 women suffer a decrease in fertility that declines abruptly after their 34th year of age (Silber et al., 2017). The decrease of mitochondrial DNA during life contributes to the loss of mitochondrial functions. This will age the ovarian tissue, and consequently it will affect the oocyte quality and the fertilizations's outcome (Igarashi et al., 2005; Liu et al., 2017). For men, 40 years of age is considered an advanced paternal age, where the spermatozoa suffer several mitotic divisions causing DNA fragmentation and an increased frequency of deleterious mutations (Brandt et al., 2019).

The question of the decline of fertility raises many problems, one of them being due to different definition of infertility used in several studies, as well as different methods of acquiring data (clinical services, census) (Baylis, 2014). Currently, there are two types of infertility: the first one consists of unprotected sexual intercourse for more than 5 years without being able to conceive a child and the second one is characterized by a couple with a child but uncapable of conceiving a second child after 5 years of unprotected sexual intercourse (Sun et al., 2019)

These different infertilities have different geographic incidences. The first one has been more prevalent in Africa as the second one has been more reported over Europe. Yet when we speak about infertility on a global scale, the developed countries have different distributions. For instance, Europe and USA have 15% of infertility couples and China has 25%. In developing countries, the data is even more unprecise due to the lack of hospitals facilities or absence of medical assistance for these types of situations, resulting in a false low occurrence of infertility problems (Sun et al., 2019).

Despite all the difficulties of having trustworthy data, the report of Sun et al. (2019) defends the rising of infertility, as well as of the search for infertility treatments worldwide.

Since the birth of the first *In Vitro* Fertilization (IVF) baby in 1978, technologies in reproductive medicine, allowed more than 5.4 million families to overcome infertility problems, and currently, per year, 400.000 babies are born through IVF, representing a percentage of 3% in the developed countries. However, these technologies are expensive, mostly because of the low rate of success of these protocols, forcing repeated attempts (Luoma, 2005; Magli et al., 2008; Duranthon & Chavatte-Palmer, 2018)

Animal models:

The research dealing with human health issues raises questions related to ethical problems if the study uses human samples or individuals. Therefore, it is necessary to use animal models, although it has its good and bad points. For instance, in the reproductive field, animal models allow an analysis of a higher number of embryos, frequently without ethical problems. On other hand, the results acquired need to be carefully evaluated in order to be transposable to humans, due to interspecies differences.

Assisted Reproductive Technologies (ART), addressed to infertility, a health problem, benefit as well of animal models. These models have contributed to the progress in the knowledge of the embryo´s molecular and cellular mechanisms and the correction of methodologies (Duranthon & Chavatte-Palmer, 2018).

Despite rodents being the most commonly used model for studying human diseases and physiology, due to their cost, effectiveness, and short generation time, rodents have some barriers in particular when dealing with infertility problems and ART. For instance, folliculogenesis studies in rodents are an example of a limitation incapable to transpose, due to rodents being poly-ovulatory while women are mono-ovulatory. Other limits are owed to differences in the length of cycles (4 days, mouse; 28 days, woman) and gestation (21 days, mouse; 9 months, woman) (Soncin et al., 2015; Paixão et al., 2017; Abedal-Majed & Cupp, 2019). These disadvantages

prompt to the need of finding other animal models. Animals such as pigs are studied for anatomical reasons (Bassols et al., 2014), sheep for the physiological and gestational monitorization (Padmanabhan & Veiga-Lopez, 2013), and in turn, the bovine model is helpful to understand reproductive disorders in man and woman. Cow and woman have a similar ovulatory cycle, gestation length, follicular growth, and both species are mono-ovular which is useful to study follicle and oocyte development (Adams & Pierson, 1995; Adams et al., 2012; Abedal-Majed & Cupp, 2019).
In the present assays, the Bovine specie was chosen as the animal model because it is an attractive model for human reproduction (Mattern et al., 2016). Several studies have used bovine as the animal model to study reproduction (reviewed in Malhi et al., 2007) and other studies as Agarwal, et al. (2006) and Leite et al. (2018) studied the oxidative stress in *in vitro* produced cattle embryos.

Another point of view to study bovine is the current commercialization of bovine embryos worldwide. IVF was introduced as an experimental procedure in cattle in 1981 and nowadays, more than 900,000 bovine embryos are produced per year (Perry, 2016). And 50% are produced with a similar protocol to humans (Baruselli et al., 2015). These industries need a technique optimization, due to the low rates of success, where merely 30% to 40% of collected oocytes can successfully go through cleavage and reach the blastocyst stage (Fair et al., 2001; Rizos et al., 2008; Pereira & Marques, 2017; Yang et al., 2017). Also, for humans, it will be favourable to understand how these procedures affect cellular physiology, during embryo development (Springer et al., 2021) and also its repercussion on foetal, neonatal, and adult physiology (Hansen, 2020).
Another aspect to consider is the economic point considering the ease of obtaining bovine ovaries. There is no need to breed cows or bulls because they are already in the meat market. Taking out some finances charges of the project. The ovaries used in the present study were collected in a local slaughterhouse and the thawed sperm from INIAV'S stock, from a bull with sperm of good quality.

Techniques:

Over the past decades, ART has been evolving, with new treatments and protocols (Inhorn & Patrizio, 2014). The process begins with a medical examination and an endocrine study of the couple, which will contribute to identify the reproductive problem and at what stage of germ cell development or fertilization happens (Trounson & Gardner, 1993; Ferlin & Foresta, 2020). The woman is submitted to an ovarian stimulation for oocyte collection (Gameiro, 2016; Springer et al., 2021), and according to the diagnosis, the implemented treatments may be Gamete Intrauterine Transfer (IT), *In Vitro* Fertilization (IVF) our Intracytoplasmic sperm injection (ICSI) (Gameiro, 2016).

The technique utilized in this work was IVF. The treatment starts with ovarian stimulation using hormones with the purpose to generate more oocytes than usual. Then it will proceed by oocyte collection, insemination, embryo culture, and embryo transfer (in a blastocyst stage) to the uterus of the mother (Laverge et al., 2001; Glujovsky et al., 2012, 2016; Pandian et al., 2015; Li et al., 2021)

IVF is not the method of choice nowadays, because ICSI has been increasingly practised replacing IVF. Especially in cases of male infertility problems but used in all the circumstances, due to the efficacy and standardization of the method (Esteves et al., 2018; Bedoschi et al., 2020). However,

in non-male infertility problems evidence of preference between the two methods do not exist, and in cases of unexplained infertility, IVF has higher rates of success (Palermo et al., 2015; Esteves et al., 2018), as well as when IVF is compared with IT (Pandian et al., 2015). Out of the comparisons between methodologies and analysing the rates of success of IVF, just 70% of the oocytes fertilized undergo the first cleavage, and of those only 20-50% can achieve the blastocysts stage (Agarwal et al., 2005). The percentage of pregnancies coming to an end is solely 30% (Truong et al., 2016), indicating an unoptimized procedure, probably due to technical problems allied to the difficult resolution of cases dealing with infertile couples (Kageyama et al., 2021).

Media and the conditions during IVF tried to resemble the environment of the reproductive tract (Trounson & Gardner, 1993; Thompson et al., 2000; du Plessis et al., 2008; De Munck et al., 2019). However, it is extremely complicated to mimic such a complex and mutable condition as it is in vivo (du Plessis et al., 2008). Embryo culture conditions have different impacts on the development, both in animals and humans (Duranthon & Chavatte-Palmer, 2018). Frequently problems may occur due to temperature changes or air quality problems (Ombelet & Onofre, 2019). To appease those negative effects, it is necessary to maintain the embryos at 38.5º C and to subject the culture to 5% of O_2, during the developmental phase (Steptoe et al., 1971; De Munck et al., 2019) in order to mimic the conditions of the reproductive tract, which is characterized by low levels of oxygen (2-8% O2) (Fischer & Bavister, 1993; Truong et al., 2016). The fluids from the Fallopian tubal and uterus are as well mirrored by the media enriched with nutrients, such as pyruvate, glucose, amino acids (Tay et al., 1997), and an antioxidant complex (Fischer & Bavister, 1993; Truong et al., 2016).

Indeed, over time antioxidants found in the Fallopian tube and in the uterus, such as Superoxide dismutase (SOD), Catalase (CAT), glutathione S-transferases (GST), and Glutathione peroxidase (GPx), were added to the culture media used in ART (Divyashree & Yajurvedi, 2018). However, this type of antioxidant enzymes is a very expensive method (Guérin et al., 2001). There are non-enzymatic antioxidants in the tubal fluids, like hypotaurine and taurine, which are supplied in some protocols, as well as glutamine, that is very important amino acids in the intercellular pool of antioxidants (Burton et al., 2003; Truong et al., 2016; Yang et al., 2017). Unfortunately, some of these substances are membrane impermeable, failing in one of the most important purposes, to eliminate reactive molecules inside of the bigger source, the mitochondria (Burton et al., 2003; Szeto, 2008; Guzman-Villanueva & Weissig, 2015).

ROS:

Mitochondria generate ATP on aerobic conditions through oxidative phosphorylation (OXPHOS), which is essential for the main activities in the cell (Munro & Treberg, 2017; Kausar et al., 2018; Schofield & Schafer, 2021). The transformation of NADH to ATP is a process that includes 5 protein complexes in the inner mitochondrial membrane and a transmembrane electrochemical gradient (Figure 1). Between complexes an electron transport chain (ETC) happens, which leads sometimes to electron slippage, generating Reactive Oxygen Species (ROS) (Sousa et al., 2018; Schofield & Schafer, 2021).

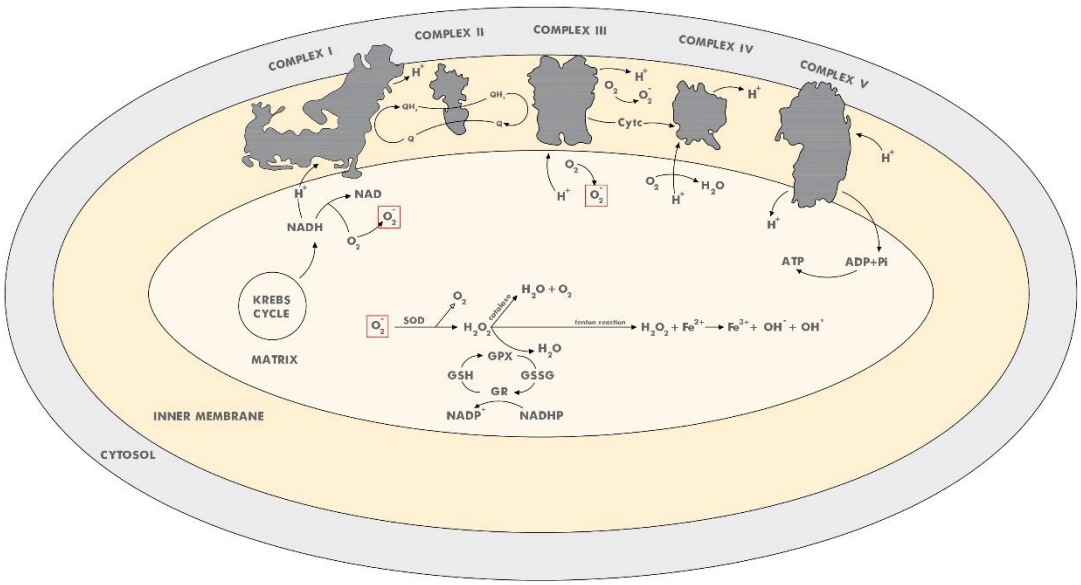

Figure 1 Scheme of a mitochondrion showing the electronic respiratory chain, and the reaction that give rise to free radicals. The mitochondrial respiration chain in the Inner Membrane uses NADH that is produced in Krebs Cycle. The reduction of NADH to NAD is responsible for the donation of H^+ in the complex I. Complex I, III and IV pump protons across the Inner Membrane, which flow back into de matrix via the ATP synthesis, driving the continued production of ATP. The reduction of NADH is also responsible for the release of O_2. In the oxidative phosphorylation, O_2 is partially reduced to generate H_20 (complex III). One of the products of this operation is O_2, a free radical (marked in a red square). This first free radical of the electronic respiration chain if not synthetized for SOD will be part of the Fenton reaction, generating OH^- (Marín-García, 2012; Sousa et al., 2018).

ROS **c**an be derived from complex I and III, as a spontaneous ETC leakage, where a reactive anion, Superoxide ($O_2^{\cdot -}$) is generated. In physiological conditions, natural ROS exists in the body being responsible for few signals transduction (Sena & Chandel, 2012; Schofield & Schafer, 2021), influencing GSH pool, activation of mitophagy (Scherz-Shouval & Elazar, 2011; Mailloux & Treberg, 2016; Munro & Treberg, 2017), boosting differentiation and involved in processes such as the immune and inflammatory response (Kishida & Klann, 2007; Veal et al., 2007; Grimm & Eckert, 2017). Nonetheless, the level of ROS due to its cytotoxic effects is controlled by endogenous antioxidants, namely SOD, that is responsible to transform $O_2^{\cdot -}$ in a least reactive molecule, the hydrogen peroxide (H_20_2) that in turn is converted by CAT or GPX in H_20 and stable O_2 (Figure 1). However, when this defence cannot neutralize the high levels of ROS, some problems may appear (Grimm & Eckert, 2017; Schofield & Schafer, 2021). This deregulation is known as Oxidative stress, one of the biggest problems for ART success (du Plessis et al., 2008; Ramalho-Santos et al., 2009; Leite et al., 2018), steering to DNA, protein, carbohydrates oxidation, and lipid peroxidation. This damage in the mitochondria will generate a vicious cycle in the production of ROS, often ending in cell death (Grimm & Eckert, 2017; Schofield & Schafer, 2021).

Mitochondriotropic Antioxidants:

Several antioxidants have been used to control oxidative stress in different cells. These molecules may have different chemical structures and targets within the organisms. In fact, through the last decade, other antioxidants more suitable to eliminate ROS generated within the cell were adopted. For instance, the natural antioxidant, melatonin (Zhang & Zhang, 2014) has the advantage of being a target molecule to the inner cell mass (He et al., 2016), achieving good results in the reduction of H_2O_2 and OH^-, as well as in the improvement of the mitochondrial functions and the *in vitro* maturation rate of oocytes in several species (Poeggeler et al., 1994; Nakamura et al., 2003; He et al., 2016; Yang et al., 2017). On the other hand, MitoQ, which is a synthetic ubiquinone, is probably the furthermost studied antioxidant-targeted to mitochondria that due to the delocalized lipophilic cation, triphenylphosphonium (TPP), a nanocarrier, can permeate in the negative lipid bilayer of the mitochondria being oxidized in complex II (Mcmanus et al., 2011). Although complex II is not the epicentre of ROS production, MitoQ is useful in preventing peroxidation by eliminating Lipid Peroxyl (LOG) and Alkoxyl (LO) radicals (Ernster et al., 1992; Teixeira et al., 2017b). However, MitoQ causes toxicity on the mitochondrial bioenergetic apparatus, even at low concentrations (Teixeira et al., 2017b), and cannot contribute to the inhibition of iron toxicity (Guzman-Villanueva & Weissig, 2016; Teixeira et al., 2017a). This last process is due to the metal iron present in the cell that takes part in the Fenton reaction ($H_2O_2+Fe^{2+} = Fe^{3+}+OH^-+OH^*$) responsible for the production of the most reactive radical, the OH^-, which is the principal answerable for oxidative damage (Schofield & Schafer, 2021).
Moreover, there are other therapeutics for oxidative stress in mitochondria based on vitamins and metabolic cofactors, such as ascorbate, L-carnitine, folic acid, and CoQ10. However, these molecules have some associated problems due to the high values of toxicity, lack of target, or impairing mitochondrial physiology (Marín-García et al., 2013). Therefore, novel therapeutics need to be tested in order to minimize ROS, especially when artificially produced during *in vitro* fertilization techniques (Kageyama et al., 2021).

AntiOxCIN4 e AntiOxBEN2:

Currently, different authors developed several scaffolds inspired by dietary antioxidants as promising strategies to target excessive ROS production (Teixeira et al., 2018). This strategy was used to help the embryo development improving its surrounding environment through the administration of antioxidants that exist in the diet. Vitamins C and E have demonstrated good results either in male (Sharma & Agarwal, 1996; Ruder et al., 2009) or in female infertility especially Vitamin E, being a chain breaking in the process of lipid peroxidation (Buettner, 1993; Burton et al., 2003). Moreover, antioxidants derived from plants have gained increasing importance in the last years derivative of their good results and production facility (Zhang & Demain, 2005; Martino et al., 2016). Experiences were done during the oocyte maturation with derivates of phenol acid: Verbascoside, present in *Aloysia Citrodora* and *Olea Europea* (du Plessis et al., 2008; Ruder et al., 2009; Martino et al., 2016) and with the two antioxidants AntiOxCIN4 and AntiOxBEN2, synthesized from caffeic acid and gallic acid respectively (Teixeira et al., 2020).

Previous work from Santos (2020), studied the oxidative stress levels in the male germline and accomplished promising results by the supplementation of AntiOxBEN2 in the capacitation medium of the spermatozoa and in the fertilization medium, showing that this antioxidant could provide a better environment for embryo production.

ROS formation is notable during gametogenesis and fertilization processes (Nasr-Esfahani et al., 1990; Ramalho-Santos et al., 2009; Truong et al., 2016), but the embryos are the major sources of ROS since they have the fastest developmental rate (Truong et al., 2016). Mitochondrial oxidative phosphorylation needs to produce energy for those essential developmental events, such as the first cleavages, genome activation, morula compaction, and the formation of the blastocyst (Rizos et al., 2002), emphasizing the need to control oxidative stress levels at this stage of reproduction (Mulla et al., 2018).

According to Troung et al. (2016), benefits were obtained by the addition of antioxidants at early phases of embryo development improving cleavage and blastocyst rates. Changes in the culture medium can, therefore, optimise embryo quality, as well as the ratio of success on embryo production and implantation (Mulla et al., 2018).

In the present work, we have tested, for the first time, AntiOxCIN4 and AntiOxBEN2 supplementation to the embryo culture medium, in order to study their effects on their developmental competence and, mitochondrial function. Cleavage and blastocyst embryo rates and their quality as well as the number of viable embryos obtained for transfer both in fresh and after vitrification were assessed, comparing the two drugs and in different concentrations (1 µM, 2.5 µM, 10 µM). Those concentration were chosen based on the results of the articles from Teixeira et al., 2020 and Santos, 2020. The two antioxidants are a novel therapeutic strategy developed by our team for the elimination of ROS in the mitochondria.

Although natural antioxidants are an excellent way to approach the problem, it has yet a limited rate of incorporation, as only a small fraction can penetrate organelles (Teixeira et al., 2012) such as the mitochondria. To solve this problem a carbon chain (lipophilic spacer) and a TPP was added to the two natural derived-antioxidants (Teixeira et al., 2012; Guzman-Villanueva & Weissig, 2016) that allowed the drugs to penetrate and accumulate specifically in the mitochondria, creating a mitochondriotropic molecules (Teixeira et al., 2012; Guzman-Villanueva & Weissig, 2016). Therefore, AntiOxCIN4 and AntiOxBEN2 are free radical scavenging preventing lipid peroxidation. They have the advantage of operating in the electron transfer chain without competing with the mitochondria chain unit, owing to their spontaneous hydrogen donation capacity at the ortho position in the catechol ring, to the peroxyl radicals, proprieties of the phenol group. AntiOxCIN4 and AntiOxBEN2 show low toxicity on mitochondrial morphology and function, as well as the property of iron-chelating through methoxylation and the donation of a hydrogen (Son & Lewis, 2002; Figueroa-Espinoza & Villeneuve, 2005; Teixeira et al., 2012, 2017; Teixeira et al., 2017, b).

These two molecules in spite of having identical antiradical activity have different efficiencies, caffeic acid has a stronger activity than gallic acid (Kikuzaki et al., 2002; Teixeira et al., 2012) but emerging both as potential candidates for first-class drugs with therapeutic application in the mitochondrial oxidative stress (Teixeira et al., 2017b, 2018).

Nowadays, oxidative stress in vivo is also a topic of utmost importance. Pregnancies occur later than ever before in a life of a couple, generating increased levels of oxidative tension, as well as higher levels of ROS (Trevizan et al., 2018). These stress levels cause a delay in embryonic development, such as in the first cleavages, responsible for DNA fragmentation, alteration of calcium homeostasis and ATP production, genome activation, epigenetic reprogramming, damage in cellular structures, altered membrane fluidity and cellular differentiation during the

formation of the inner cells mass and trophectoderm, and apoptosis in early stages of embryonic development (Leite et al., 2018; Soto-Heras & Paramio, 2020). Nonetheless, higher levels of ROS were found *in vitro* than *in vivo* (Luvoni et al., 1996; Nohalez et al., 2018), which is caused by the manipulation of oocytes, sperm, and embryos in ART, also influenced by lights, nutrient imbalance, growth factors, pH and osmotic shocks (Guérin et al., 2001; Leite et al., 2018).

Although as referred, adding an antioxidant to a culture medium is an old intricate problem, the abovementioned properties of antioxidants target to mitochondria open a window to control excessive ROS production frequently linked to several infertility problems. However, many questions remain unanswered in this methodology regarding not only which ROS scavenger to use but also its concentration and the phase of development to implement it (Guérin et al., 2001; Santos, 2020; Teixeira et al., 2020).

Methodology:

Chemicals and reagents

Cell culture medium, water for embryo culture, media components, chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified.

Synthesis of AntiOxCIN4

The synthetic strategy and procedures used in the synthesis of the mitochondriotropic antioxidant AntiOxCIN4 (Figure 2) have been previously described in Teixeira et al. (2017). Briefly, dimethoxycinnamic acid was linked to an alkyl spacer, that influences the charge-carrier mobilities of the molecule. The terminal group was sifted to triphenylphonium cations to the molecule turn into a lipophilic compound and finally, it was demethylated by boron tribromide for prevent the formation of peroxides.

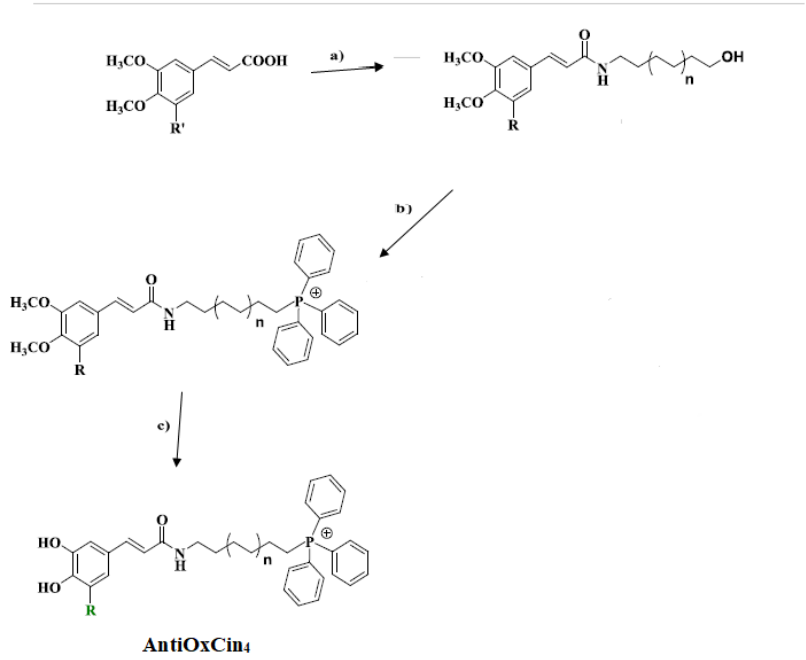

Figure 2 Synthetic strategy used for production of mitochondriotropic antioxidant based on caffeic acid (AntiOxCIN4). Reactions a) linked with an alkyl spacer; b) conversion of alcohol (OH) to a cation (P+); C) Demethylation. Adapted image from Teixeira et al., 2017,a

Synthesis of AntiOxBEN2

The synthesis of the AntiOxBEN2 (Figure 3) was similar to the synthesis of AntiOxCIN4, with a difference of the initial compound. AntiOxBEN2 drift of trimethoxybenzoic acid, which was linked to an alkyl spacer, the terminal group sifted to triphenylphonium cation and finally was demethylated.

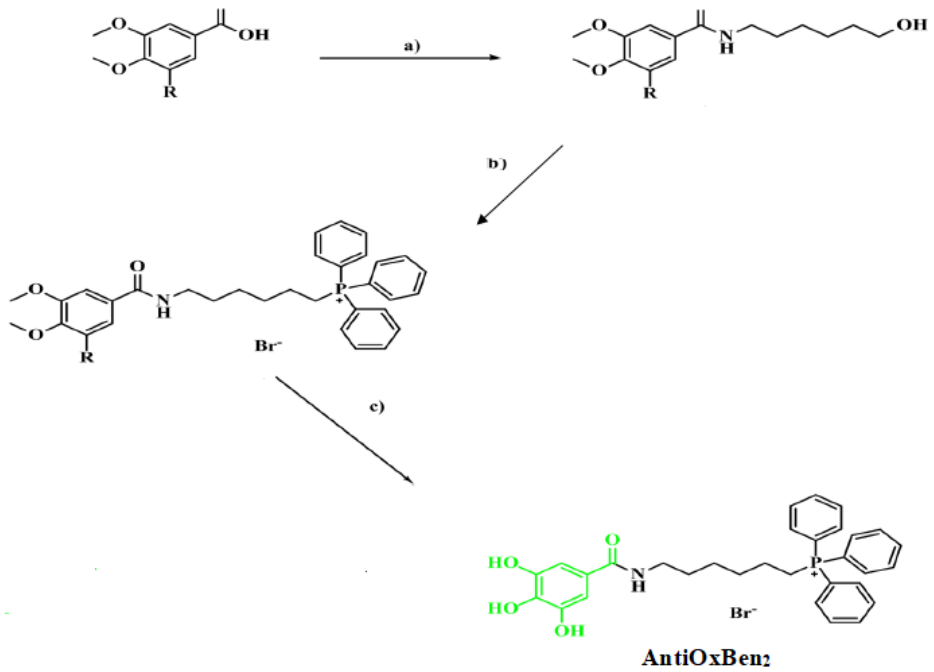

Figure 3 Synthetic strategy used for production of mitochondriotropic antioxidant based on gallic acid (AntiOxBEN2). Reactions a) linked with an alkyl spacer; b) conversion of alcohol (OH) to a cation (P+); C) Demethylation. Adapted image from Teixeira et al. (2017,b)

Experimental design

To study the effects of the antioxidants AntiOxCIN4 and AntiOxBEN2, in the embryonic development and levels of ROS, three major assays were performed. The effects of the antioxidants were examined in fresh and frozen bovine embryos, due to the increased practice of transferring cryopreserved-warmed embryos to the mothers, in the current clinic (Wong et al., 2021).

For the first and second experiments, a total of 3443 oocytes were collected during 6 and 8 sessions, respectively. The oocytes were kept in the maturation medium for 22h and inseminated with frozen-thawed semen (Day 0 = D0). After 18h of co-incubation, the presumptive zygotes were randomly distributed into the groups (1437 and 2006 in the first and second experiments, respectively).

In the first experiment, the embryo developmental medium was supplemented with AntiOxCIN4 and in the second experiment, the medium was supplemented with AntiOxBEN2. For the first assay four groups were constituted: Control, 1CIN, 2.5CIN, 10CIN (corresponded to 0 µM, 1 µM, 2.5 µM, and 10 µM of AntiOxCIN4). For the second experiment the same groups were formed but with the second antioxidant: Control, 1BEN, 2.5BEN, 10BEN (corresponded to 0 µM, 1 µM,

2.5 µM, and 10 µM of AntiOxBEN2 in the embryo culture medium). These two assays had the aim to achieve the best concentration of both antioxidants, to be recommended for embryo production.

In each experiment, the embryo development was evaluated on days 2, 7, and 9. On days 2 and 7, the analysis was only morphological and on day 9, successfully developed embryos were further analysed for their cell viability, mitochondrial membrane potential (ΔΨm), and ROS production using Hoechst 33342 plus Propidium iodide (PI), JC-1, and CellRox dyes. The percentage of embryo viable cells were calculated in the first and second experiments, on 22 (control, n=4; 1CIN, n=6; 2.5CIN, n=4 and 10CIN, n=8) and 15 (control, n=4; 1BEN, n=4; 2.5BEN, n=4 and 10BEN, n=3) embryos respectively, dyed with Hoechst and PI. The JC-1 dye was used to analyse the mitochondrial membrane potential, in 24 (experiment 1: control, n=5; 1CIN, n=8; 2.5CIN, n=5 and 10CIN, n=6) and 15 embryos (experiment 2: control, n=6; 1BEN, n=5; 2.5BEN, n=1 and 10BEN, n=3). Finally, to estimate ROS in embryo cells, the CellRox dye was applied to 7 embryos (control, n=1; 1CIN, n=3; 2.5CIN, n=0 and 10CIN, n=3) in the first experiment and 5 embryos (control, n=1; 1BEN, n=1; 2.5BEN, n=1 and 10BEN, n=2) in the second experiment.

A third experiment was implemented to compare the best results of the two previous experiments and further test the effects of the two antioxidants elected doses on embryo cryosurvival. In this last experiment, 1147 presumptive zygotes were divided into three groups: Control, 2.5CIN, and 2.5BEN, where embryos were supplemented during culture as above, during 6 sessions. After culture, embryos were analysed at the cleavage point at 48h post insemination and on day 8 (number, developmental stage and quality). Then only embryos with adequate quality (grade 1 and 2) were vitrified (Control, n=13; 2.5CIN, n=14; and 2.5BEN, n=19). After warming, embryos were evaluated and maintained in culture for 24 hours (Control, n=9; 2.5CIN, n=6; and 2.5BEN, n=15).

After integrity and expansion evaluation total and viable cells (Control, n=5; 2.5CIN, n=3; and 2.5BEN, n=4), mitochondrial potential membrane (Control, n=4; 2.5CIN, n=3; and 2.5BEN, n=7), and levels of ROS (Control, n=4; 2.5CIN, n=3; and 2.5BEN, n=4) were measured as in the previous assays, to signal which Antioxidant had the best performance in cryopreserved-warmed embryos.

Ovary collection and oocyte selection

Bovine ovaries were collected at a slaughterhouse and kept in phosphate buffered saline (PBS) (Gibco 14040-91) supplemented with 0.15% of bovine serum albumin (BSA, Sigma A-7888) and 0.05 mg mL^{-1} of antibiotic (kanamycin) (Sigma K-4000), at 35–37 °C, in a maximum of 2 hours. At the laboratory, the ovaries were washed with PBS supplemented with 0.05 mg mL−1 of kanamycin and maintained at 37ºC.

From each ovary COC were obtained through follicular aspiration (2-6 mm follicles) with a 10 mL syringe attached to a 19G-needle (Fonseca et al., 2020). The oocytes were deposited in washed medium (Tissue Culture Medium 199, Gibco 22340-020) supplemented with 10% foetal bovine serum (FCS) and antibiotics at a temperature of 37ºC. Then the selection of oocytes for maturation were performed. Oocytes surrounded by 3 or 4 layers of compact cumulus cells and granular cytoplasm were selected (Lapa et al., 2011). The medium for this selection was composed by TCM199 medium (Tissue Culture Medium 199, Gibco 22340-020) supplemented

with FCS and sodium pyruvate.

In vitro Maturation

After the selection, oocytes were transferred to the maturation medium, TCM199 (Tissue Culture Medium 199, Sigma M-4530) with 10% FCS, 0.2 mM sodium pyruvate, antibiotics, and 10 g mL^{-1} EGF (Epidermal Growth factor, Sigma E 4127) for 22h at 38.5 ºC and 5% CO_2 (Fonseca et al., 2020).
After the period of maturation (22h), oocytes were morphologically evaluated by using a stereomicroscope. Only oocytes with a regular cytoplasm and a high expansion level of the cumulus cells were selected for insemination (Leibfried & First, 1979; Pereira et al., 2006; Penitente Filho et al., 2012).

Sperm preparation

Frozen-thawed sperm from one male already selected through its good *in vitro* fertility results (Friesian breed) were used in these experiments. Frozen semen straws were thawed at 37ºC and the Percoll method (Fonseca et al., 2021) was used for sperm capacitation. During this process, the more capacitated spermatozoa swim through the two Percoll gradients (45 and 90%) during the centrifugation process, lodging in the bottom of the tube. The supernatant was rejected. The pellet of spermatozoa was evaluated for motility and concentration in a Neubauer camera to enable an insemination with $2x10^6$ spermatozoa mL^{-1}

In Vitro Fertilization and Embryo culture

For the *in vitro* insemination, oocytes were sort in groups of 10 and placed in droplets of 40µL of fertilization medium (FERT) immersed in mineral oil and then spermatozoa were deposited in the droplets. Gametes were co-incubated for 18 to 20h (IVF = day 0) at 38.8ºC and 5% CO_2. (Fonseca et al., 2020).

Presumptive zygotes at 20h post insemination were transferred to 100 µL droplets of synthetic oviductal fluid (SOF) supplemented with amino acids, glutamine, BSA, and different doses (1 µM, 2,5µM, 10µM) of antioxidant molecules AntiOxBEN2 and AntiOxCIN4 or without antioxidant (control) and were washed to remove cumulus cells. Subsequently, zygotes were transferred into 25 µL droplets of the same medium, layered with mineral oil, according to the experimental design, cultured at 38.8 °C in a humidified atmosphere with 5% O_2, 5% CO_2, and 90% N_2 (Fonseca et al., 2020).

After 48h of insemination, cleavage rate was assed (cleaved embryos per inseminated oocytes) and the embryos transferred to 25 µL of embryo culture medium (SOF, BSA, 10% FCS plus antioxidant or not, covered by mineral oil) to continue their development for the next 7/9 days until morphologic evaluation or cryoperservation. Then embryos were morphologically classified according to the stage of development (morula, young blastocyst, blastocyst, expanded blastocyst or hatched) and quality (Grade 1 – Excellent/Good, Grade 2- Fair, Grade 3 – Bad, following the International Embryo Technology Society guidelines (Stringfellow, 1998; Santos, 2020).

Assessment of embryo viability and ROS level

The protocol for the assessment of viable cells was adapted from Romão et al. (2013). A fluorescent DNA staining dye (Hoechst 33342) was used to evaluate total cells along with Propidium iodide (PI), a stain that penetrates damaged membranes of dead cells (Choi et al., 2020; Lämmle et al., 2021). The quantification of ROS level was performed with CellRox (Thermo Fisher Scientific, USA) through the quantification of the cell-permeant dye, that exhibits fluorescence upon oxidation by ROS.

Good and fair embryos at day 9 of development (first and second experiments), were selected and transferred to SOF plus 20% FCS, washed in PBS and moved to 100µL of Pronase containing droplets for the digestion of the zona pellucida, for 3 minutes, and then to a droplet of Tyrodes medium for 1.5 minutes to finalize the removal of the zona pellucida. Finally, embryos were washed in PBS and incubated in SOF + 20% FCS plus 5 mM CellRox (75 µL droplets) for 1 hour in the dark, at 38.8ºC in a humidified atmosphere with 5% O_2 and 5% CO_2. Afterwards embryos were fixed in a 4% paraformaldehyde solution per 1 hour and then transferred to a solution of 50 µg mL^{-1} of Hoechst 33342 and 5 µg mL^{-1} of PI in SOF+ 20% FCS for 30 minutes. Ultimately, 2 µL of Mowiol (Caalbiochen 475904) were placed on a glass slide with each stained embryo and covered with a coverslip. Cultured cryopreserved-warmed embryos were also evaluated using the same technique (third experiment).

The mounted slide was kept in the dark and refrigerated for a couple of hours being observed in a Florescence Microscopy (Olympus BX51), using the ultraviolet, red and blue channels, to acquire the fluorescence of the Hoechst, PI and CellRox, respectively. Images were processed by ImageJ (National Insitutes of Health, USA).

Assessment of Mitochondrial Membrane Potential

The ΔΨm is related with the different electric potential between cytoplasm and inner membrane. This differential happens due to the proton transference of the ETC to the mitochondrial membrane. When the ΔΨm is high, the ETC is transferring a lot of protons to the membrane, meaning a good production of ATP, and therefore a heathy mitochondrion. On the contrary, if there is not a sufficient production of ATP, the ΔΨm decreases. Also in unhealthy environments, the ΔΨm reduces due to H_2O_2 and oxidative stress (Sun et al., 2020).

The decrease of ΔΨm is a good indirect measurement of ROS being considered an oxidative stress indicator. The lipophilic cation florescent probe, the 5, 5´, 5´-tetrachloro-1, 1´, 3, 3´-tetraethylbenzimidazolcarbocyanine iodide (JC-1, Invitrogen) can penetrate selectively in the membrane being potential-dependent. When the membrane presents a high ΔΨm, JC-1 have a big accumulation forming J aggregates emitting red florescence, an emission with a maximum of 590 nm. Conversely, when the mitochondria have a low ΔΨm, JC-1 accumulates as a monomer emitting a green florescence, an emission with a maximum of 529 nm (Fonseca, 2020; Y. Sun et al., 2020).

At the 9th day of development, the good quality embryos that achieved, at least, the blastocyst stage were selected and incubated with 5 µg mL^{-1} of JC-1 in Hanks's solution for 20 minutes at 38.8ºC and 5% CO_2. Followed by the preparation of each embryo in a warmed slide and the observation on a Fluorescence Microscopy (Olympus BX51) with excitation wave of 450nm collected in the blue fluorescence channels (BP 470-490, objective UPlanFI 20x0.50) (adaptaded from Fonseca, 2020). Images were processed by ImageJ. The intensity of red and green fluorescence and the ratio between the two colours (r = red/green) were calculated.

Cryopreservation

For cryopreservation, the vitrification protocol adapted from Pereira et al., (2008) with glycerol and ethylene glycol (EG) as permeant cryoprotectants was used. Grade 1 and 2 embryos were selected and washed in TCM medium supplemented with 20% newborn calf serum (NBCS) previously equilibrated at 38.8ºC, 5% O_2, and 5% CO_2. Then the protocol proceeded at room temperature, in three steps as follows: 10% glycerol for 5 min, 10% glycerol and 20% EG for 5 min, and finally 25% glycerol and 25% EG for 30 s, in TCM–NBCS. The tree steps protocol was used to form a gradient for slow dehydration of the inter-cell, a crucial method to inhibit the formation of ice crystals during the freezing process (Pereira, et al., 2008; Silva, 2015). Finally, embryos were put in straws with the vitrification solution as explained in Pereira, et al. (2008), sealed and plunged into liquid nitrogen.

For warming procedures, the straws will be removed from the liquid nitrogen and warmed up first at the room temperature, and on the second phase in water at 22ºC, followed by the cut of tip of the straw to empty the embryo still in the vitrification medium and galactose into a petri dish.at room temperature. The next step was the embryo transfer to the TCM–NBCS at room temperature and then to the TCM-NBCS medium but at 38,8ºC. Embryos were evaluated for their integrity and expansion (expanded, semi-expanded, or shrunken) rate

After the warming and evaluation protocol, embryos were set in a culture medium (SOF+20% FCS) and recuperated in an incubator for 24h at 38.8ºC, 5% O2, and 5% CO2 (Pereira, et al., 2008). After the recovery time, the embryos were classified as expanded, semi-expanded, or shrunken.

Statistical analysis:

Data from embryo development and quality, and post-warmed integrity and expansion were analysed with the Proc GLIMIX from SAS (Statistical Analysis Systems, SAS Inst., Inc., Cary, NC, USA) using the binary distribution and logit as a link function. The statistic test was the Residual PL. Analysis of embryo total and viable cells, CellRox and Jc-1 data were processed with proc Mixed model from SAS using Restricted maximum likelihood (REML). The Mann-Whitney U Test was used to compare embryo expansion analysis (expansion, semi-expansion and shrunken) between groups.

The sessions were considered as random factor and the treatment as fixed factor. The results were considered statistically significant when $P \leq 0.05$.

Results:

In order to achieve the best concentration of the two novel antioxidants (AntiOxCIN4 and AntiOxBEN2) for the production of embryos it was necessary to examine their developmental ability. Firstly, the cleavage ratios were analyzed for acquiring the embryo´s capacity for the first cleavages. Afterwards, the embryonic development was evaluated at day 7 (D7) and day 9 (D9), in terms of embryonic quality and developmental ratios. In the first and second experiments, the concentrations of the different antioxidants were compared to the control group, without supplementation.

Experiment 1

From the initial 1166 presumptive zygotes that were distributed into the four groups supplemented with different concentrations of AntiOxCIN4, only 127 were able to survive until day 9 of development. Discarded embryos presented a degenerated morphology. Table 1 present all the information about the embryo's numbers and developmental ratios on the day 2, 7 and 9

Table 1 Effect of AntiOxCIN4 supplemented to the developmental medium on embryo production. The Groups correspond to four different concentrations of AntiOxCIN4 (Control, 1µM CIN, 2,5 µM CIN, 10 µM CIN). The data are presented in total sum of the 5 sessions, number (n) of oocytes/embryos and in percentage (%) as the mean of the calculations explained in the methodology ± the Standard Error Mean. D7 corresponded to seventh day of embryo development and D9 to the nineth day of development

Groups	**Presumptive**	**Cleavage**		**D7 Embryos**		**D9 Embryos**	
	zygotes	**n**	**%**	**n**	**%**	**n**	**%**
Control	295	206	70.4± 3.32	32	15.5±2.60	28	13.8±3.03
1CIN	256	205	80.2±2.92	43	21.0±2.94	36	17.4±3.45
2.5CIN	317	244	77.2±2.90	44	18.1±2.55	36	14.6±2.95
10CIN	298	216	73.1±3.18	37	17.2±2.65	27	12.5±2.82

Our data showed no significant effect of AntiOxCIN4 concentration on none of the embryo rates. In Figure 4, it is possible to have a good perspective of these results, where all the groups had similar progresses through development. Although no significant differences were identified between groups, a trend (P=0.086) for an effect of AntiOxCIN4 on cleavage rate were detected. The 1CIN and 2.5CIN groups had a cleavage rate of 80.2% (P=0.02) and 77.2% (P=0.08), respectively, compared to 70.4% in the control group that although without statistical significance may have some relevance in the embryo production results.

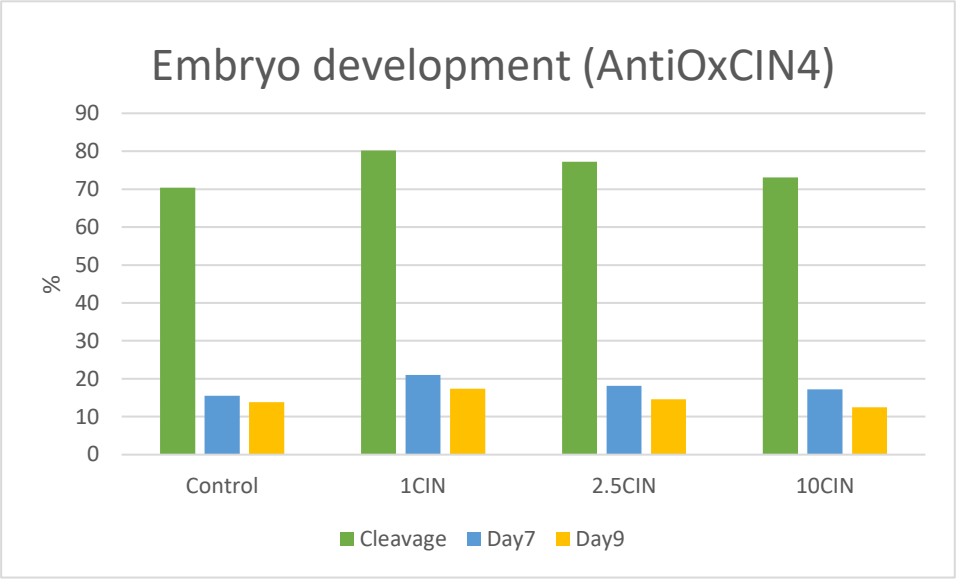

Figure 4 Effect of different concentrations of AntiOxCIN4 (Control, 1µM CIN, 2,5 µM CIN, 10 µM CIN) supplemented to the developmental medium on embryonic developmental rates at Day 2 (cleavage), Day 7 and Day 9. Results presented in percentage.

For a future successful pregnancy, the analysis of embryo cellular divisions and their appropriate development at each day, evaluated through the developmental stages as well as embryo quality, are of primordial importance.

For a proper embryo development, it would be expected to observe Morulae until Blastocyst stages at day 7 while at day 9 Expanded Blastocyst to Hatched Blastocyst would prevail. Our data followed this pattern (Figure 5 and 6), not revealing a statistically significant higher speed in the embryo development or embryo numbers between treatments.

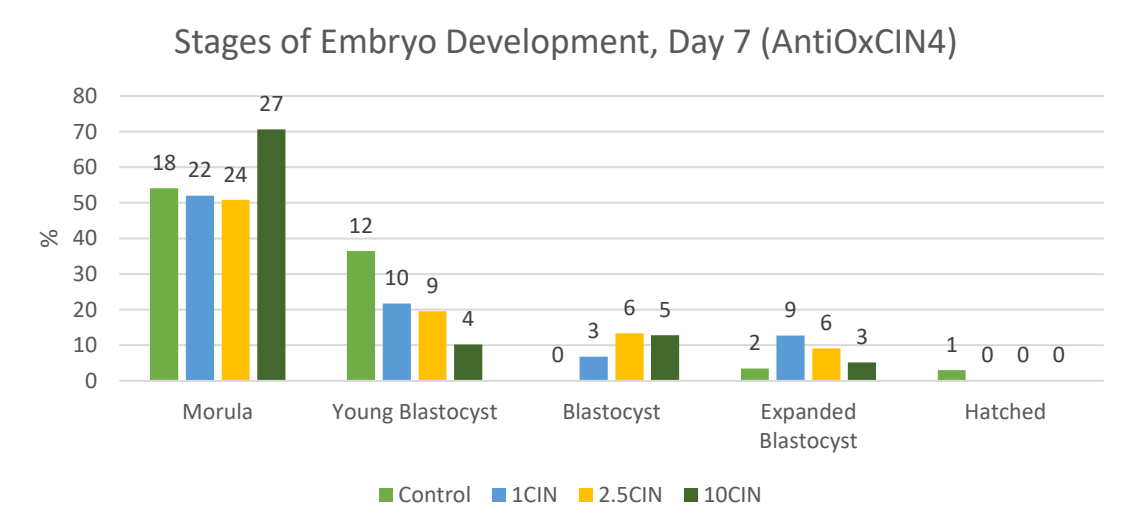

Figure 5 Effect of different concentrations of AntiOxCIN4 (Control, 1µM CIN, 2,5 µM CIN, 10 µM CIN) on the different stages of embryo development, assayed at Day 7. Results presented in percentage and the numbers above bars represent total embryos at each stage and group.

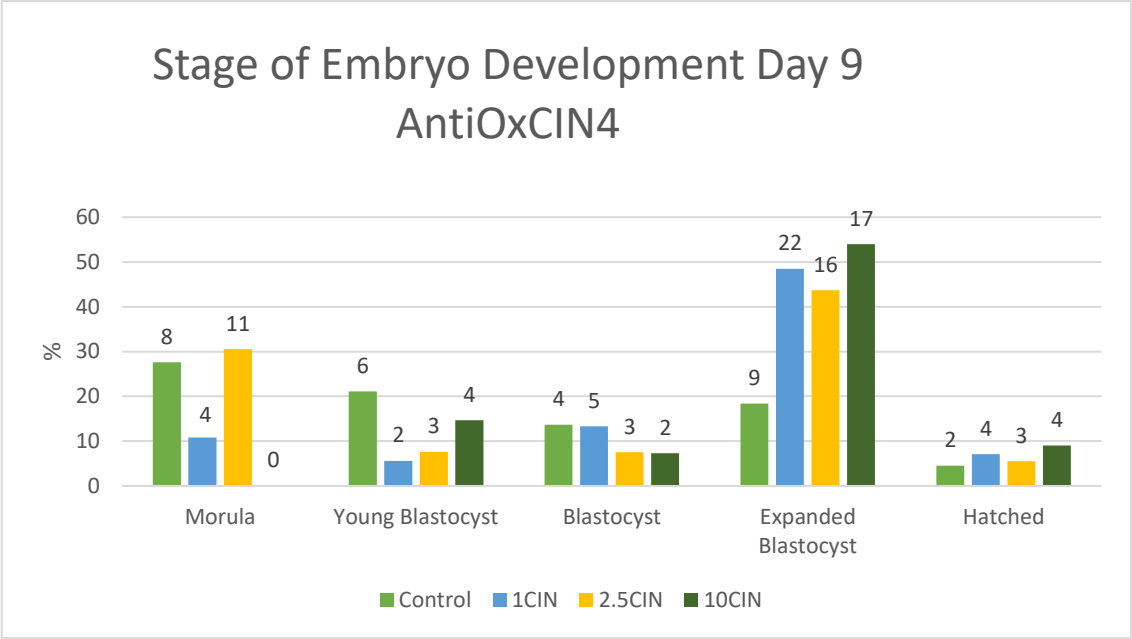

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Figure 6 Effect of different concentration of AntiOxCIN4 (Control, 1µM CIN, 2,5 µM CIN, 10 µM CIN) present in the developmental medium on different stages of embryo development, assayed at Day 9. Results presented in percentage and the numbers above the bars represent total embryos at each stage and group.

A relevant effect (P=0.037, Figure 7) of the antioxidant AntiOxCIN4 supplementation on embryo quality was identified. The 2.5CIN (7.8%, P=0.031) and 10CIN (1.5%, P=0.012) groups had fewer embryos with bad quality than the Control group (30.5%)

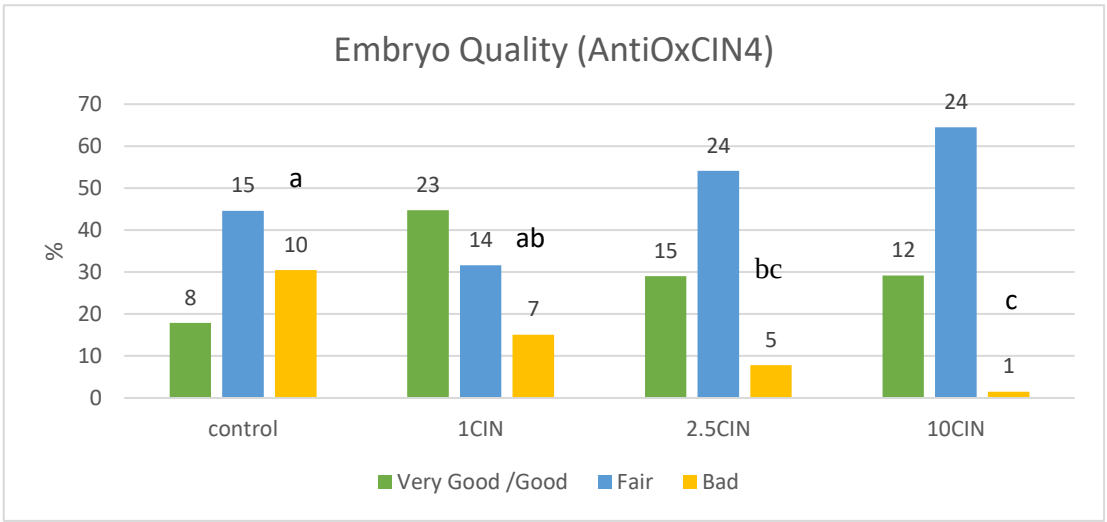

Figure 7 Effect of different concentration of AntiOxCIN4 (Control, 1µM CIN, 2,5 µM CIN, 10 µM CIN) present in the developmental medium on the quality of produced embryos. The embryo quality was classified based on morphological criteria (Very Good/Good, Fair and Bad). Results are presented in percentage and the numbers above bars represent total embryos at each stage and group. Different letters indicate statistical differences between groups.

Assessment of embryonic viable cells

After the morphological evaluation, an assay using two dyes, PI and Hoechst was implemented to calculate the percentage of viable cells in each embryo. The aim of this analysis was to further test the embryos showing good stages of development and quality, as well as to verify the accuracy of the morphological evaluation.

As shown in table 2, the percentage of viable cells of the control group is the lowest (27.8%) while the 2.5CIN group had the highest percentage (70.3%) but these results were not significantly different.

Table 2 Assessment of embryo total and viable cells after culture with different doses of AntiOxCIN4. The total cell number were assessed with Hoechst dye, the number of dead cells through Propidium iodate, and the percentage of Viable cells estimated in the four groups Control, 1CIN, 2.5CIN, and 10CIN, at day 9 of embryonic development. Data are express as averages ± SD (Standard Deviation)

GROUPS	N	TOTAL CELLS (N)	DEATH CELLS (N)	VIABILITY %
CONTROL	4	63±15.3	47.5±16.3	27.8±14.7
1CIN	6	101.6±15.3	34.6±14.5	60.3±13.4
2.5CIN	4	94.5±17.1	28.3±16.3	70.3±14.7
10CIN	8	113.1±12.1	67.5±11.5	40.2±11.0

Assessment of ROS level

To inquire embryonic ROS levels, a new protocol using a dye named CellRox was implemented in the laboratory and then the fluorescence intensity was measured in the embryos. Unfortunately, this assay began to be assembled only in the middle of the experiment. For this reason, we had only 7 embryos in this assay and none in one of the most important groups of the experiment, the 2.5CIN (Table 3). Therefore, it was impossible to perform a statistical analysis of the data in this assay. The two groups 1CIN and 10CIN obtained similar results to the Control group (Table 3).

Table 3 Assessment of Ros level in embryos cultured in medium supplemented with different doses of AntiOxCIN4, analysed by using CellRox dye. The CellRox intensity was calculated as an average according to the embryo number available at day 9 of development in each group, Control, 1CIN, 2.5CIN, and 10CIN. Data are expressed as averages or at the only value in the case of the control group.

GROUPS	N	CELLROX INTENSITY
CONTROL	1	24.5
1CIN	3	24.8
2.5CIN	0	-
10CIN	3	23.6

Assessment of Mitochondrial Membrane Potential

The ΔΨm was analysed to understand the effects of the supplementation of AntiOxCIN4 (Table 4) on the functionality of the embryo´s mitochondria. The assessed of the ΔΨm was performed through the ratio of the measured red (high ΔΨm) and green (low ΔΨm) fluorescence after staining the embryos with JC1.

Table 4 Assessment of the Mitochondrial Membrane Potential on embryos cultured in a medium supplemented with different doses of AntiOxCIN4 (CIN). The ΔΨm was measured by the average of the ratios (red intensity/green intensity) on embryos at day 9 of development in each group, Control group, 1CIN, 2.5CIN, and 10CIN. Data are expressed as averages and in the last column the averages ratios ± SD (Standard Deviation).

Groups	N	Red intensity	Green intensity	Ratio
Control	5	45.4±17.0	52.8±12.4	0.76±16.6
1CIN	8	34.6±15.2	67.2±10.3	0.47±15.0
2.5CIN	5	30.7±16.4	48.2±11.7	0.62±16.0
10CIN	6	37.9±16.2	67.6±11.4	0.55±15.9

The optimum ratio would be a ratio of 100% or more, which would mean a bigger percentage of healthy mitochondria (emitting red fluorescence) than unhealthy (emitting green fluorescence). So, our expectation was to have a bigger ratio in the 2.5CIN group compared to the Control group, as suggested by the morphological evaluation. However, no significant differences were identified between groups.

Experiment 2

During this experiment, the embryo culture was supplemented with four different concentrations of AntiOxBEN2, similarly to the experiment 1. This supplementation was implemented at the initial stages of embryo development. Of the 2006 presumptive zygotes that were cultured only 128 embryos were alive at the 9th day of development (Table 5).

Table 5 The effect of AntiOxBEN2 supplemented to the developmental medium on embryo production. The Groups correspond to the four different concentrations of AntiOxBEN2 (Control, 1µM BEN, 2,5 µM BEN, 10 µM BEN). The data is presented in total sum of the 5 sessions, number (n) of embryos and in percentage (%) as the mean of the calculations explained in the methodology ± the Standard Error Mean. D7 corresponded to seventh day of development and D9 to the nineth day of development.

	Presumptive zygotes	**Cleavage**		**D7 Embryos**		**D9 Embryos**	
		N	**%**	**N**	**%**	**N**	**%**
Control	442	308	70.5±4.27	40	12.7±2.25	25	7.4±2.05
1BEN	372	248	67.4±4.58	48	19.0±3.0	35	12.6±3.1
2.5BEN	377	260	69.4±4.44	46	17.4±2.83	36	13.2±3.17
10BEN	422	270	64.7±4.65	56	20.5±3.03	32	10.6±2.69

In figure 8 it is possible to observe the developmental ratios of the embryos through the nine days of culture. The addition of the antioxidant did not have a significant influence on the embryo developmental rates at any stage. In fact, the cleavage rate and Day 7 and 9 embryo rates were very similar in all groups.

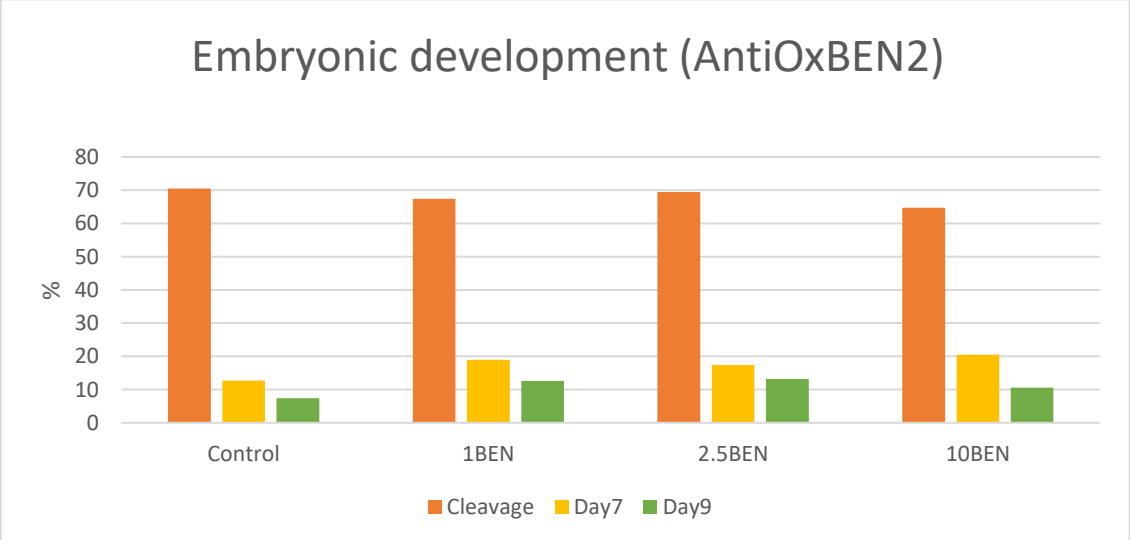

Figure 8 Effect of different concentration of AntiOxBEN2 (Control, 1µM BEN, 2,5 µM BEN, 10 µM BEN) supplemented to the developmental medium on embryonic developmental rates at Day 2 (cleavage), Day 7 and Day 9. Results presented in percentage.

Also, the velocity of the embryo development was not affected by the antioxidant supplementation neither on day 7 (Figure 9) or on day 9 (Figure 10).

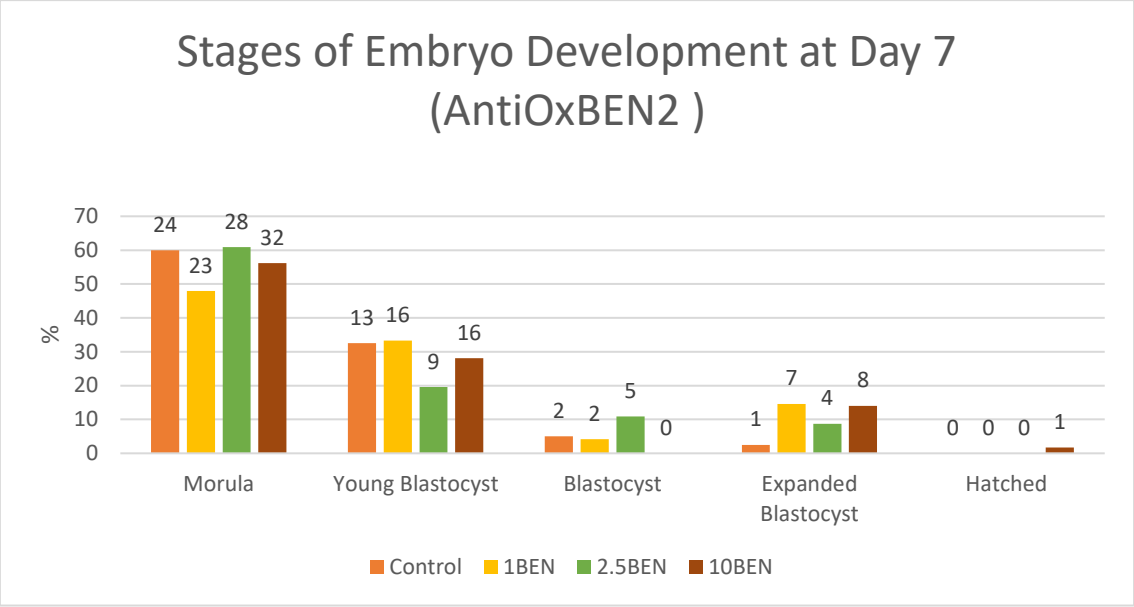

Figure 9 Effect of different concentrations of AntiOxBEN2 (Control, 1µM BEN, 2,5 µM BEN, 10 µM BEN) on different stages of embryo development, assayed at Day 7. Results presented in percentage and the numbers above the bars represent total embryos at each stage and group.

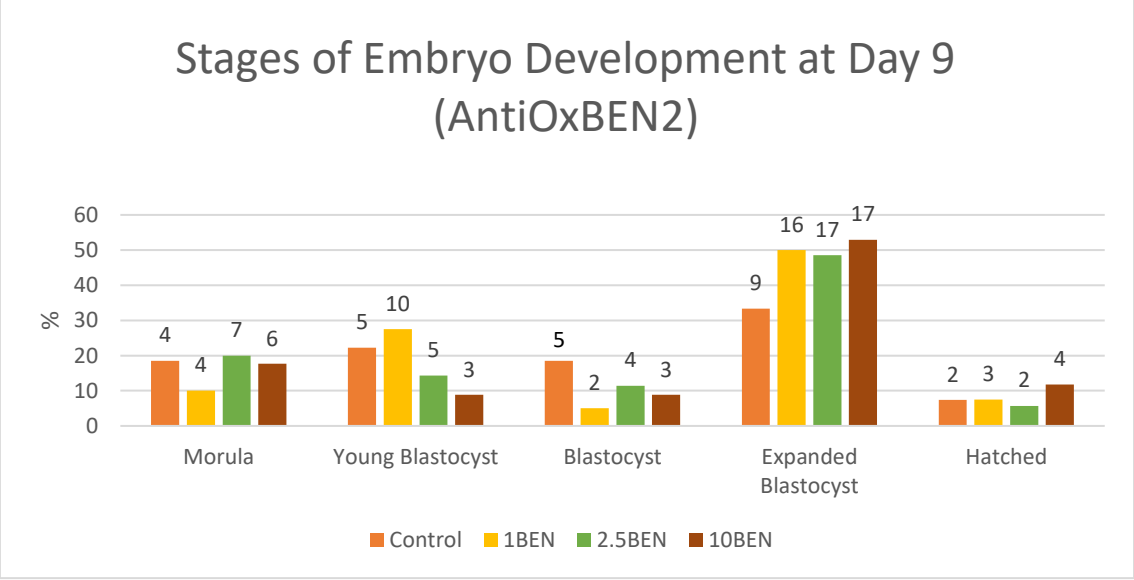

Figure 10 Effect of different concentrations of AntiOxBEN2 (Control, 1µM BEN, 2,5 µM BEN, 10 µM BEN) supplemented to the developmental medium on embryo different stages, assayed at Day 9 of the embryonic development. Results presented in percentage and the numbers above bars represent total embryos at each stage and group.

In the second experience, an important statistically significant effect (P=0.004) of AntiOxBEN2 in the improvement of embryo quality was identified. Embryos that were supplemented with 2.5 BEN dose during the development, had higher percentages of Very Good/ Good quality embryos (41.9%) when compared to control (5.7%, P=0.0014, Figure 11). The 2.5BEN group also had more very good/good quality embryos than the 10BEN (9.5%, P=0.002) and 1BEN (12.5%, P=0.008) groups.

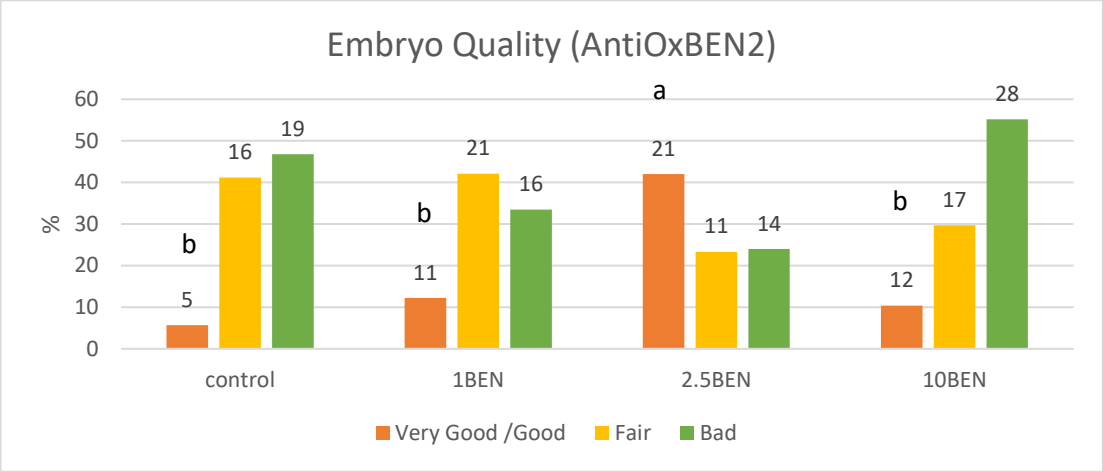

Figure 11 Effect of different concentration of AntiOxBEN2 (Control, 1µM BEN, 2.5 µM BEN, 10 µM BEN) present in the developmental medium on the quality of produced embryos. The embryo quality was classified based on morphological criteria (Very Good/Good, Fair and Bad). Results presented in percentage. Different letters indicate statistical differences between groups.

Assessment of embryonic viable cells

The results of the percentage of viable cells obtained after the supplementation of embryo culture medium with different doses of AntiOxBEN2 were represented in the Figure 12.

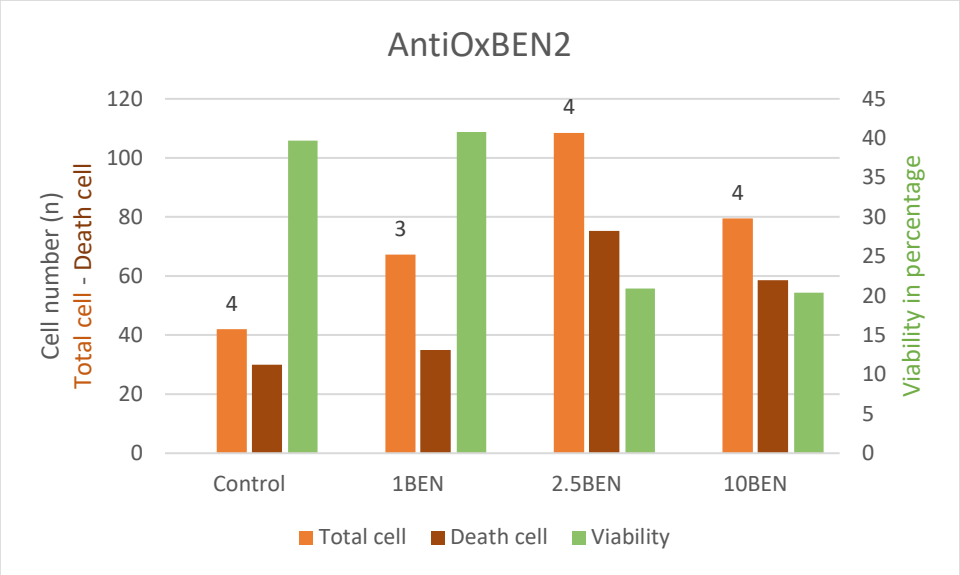

Figure 12 Assessment of total and viable cells in the embryo after cultured with different doses of AntiOxBEN2. The total cell number were assessed with Hoechst dye, the number of dead cells through Propidium iodate, and the percentage of Viable cells estimated in the four groups Control, 1BEN, 2.5BEN, and 10BEN, at day 9 of embryonic development. Results of Total cell and Death cell presented in average of embryos in each group, Viability presented in percentage (Total Cell-Death cell)/Total cell. Number above the Total cell bar are the embryos assessed in each group

No differences between groups were identified for the variables total cells, dead cells or viability. However, a tendency (P=0.07) for an effect of the antioxidant supplementation on the total number of cells was detected. In fact, the number of total cells of embryo from the group 2.5BEN was 108.5 cells (P=0.01) compared to 42 cells in the Control group. However, although without statistical differences, the analysis of the viable cells demonstrated that the best group in this experience was the 1BEN with the percentage of viable cells 40.8% followed by the control group with 39.7%, and then 2.5BEN with 20.9%.

Assessment of ROS level

As referred, this assay had the same problem on the assessment of Ros level in the first experiment, without sufficient embryos to make a statistical analysis. However, in this assay the control group demonstrated the biggest CellRox intensity (Table 6), corroborating the morphological analysis. Still these data should be further confirmed.

Table 6 Assessment of ROS level intensity on embryos cultured in a medium supplemented with different doses of AntiOxBEN2. The ROS level was measured on embryos at day 9 of development in each group, Control group, 1BEN, 2.5BEN, and 10BEN. Data are expressed as averages when possible.

GROUPS	N	CELLROX INTENSITY
CONTROL	1	24.5
1BEN	1	15.8
2.5BEN	1	16.0
10BEN	2	16.4

Assessment of Mitochondrial Membrane Potential

In the assessment of the ΔΨm (Figure 13), the 1BEN group had the greatest ratio (64.2%) as in the previous assay in the rate of viable cells. However, no significant differences between groups were identified in the red and green intensity or their ratio.

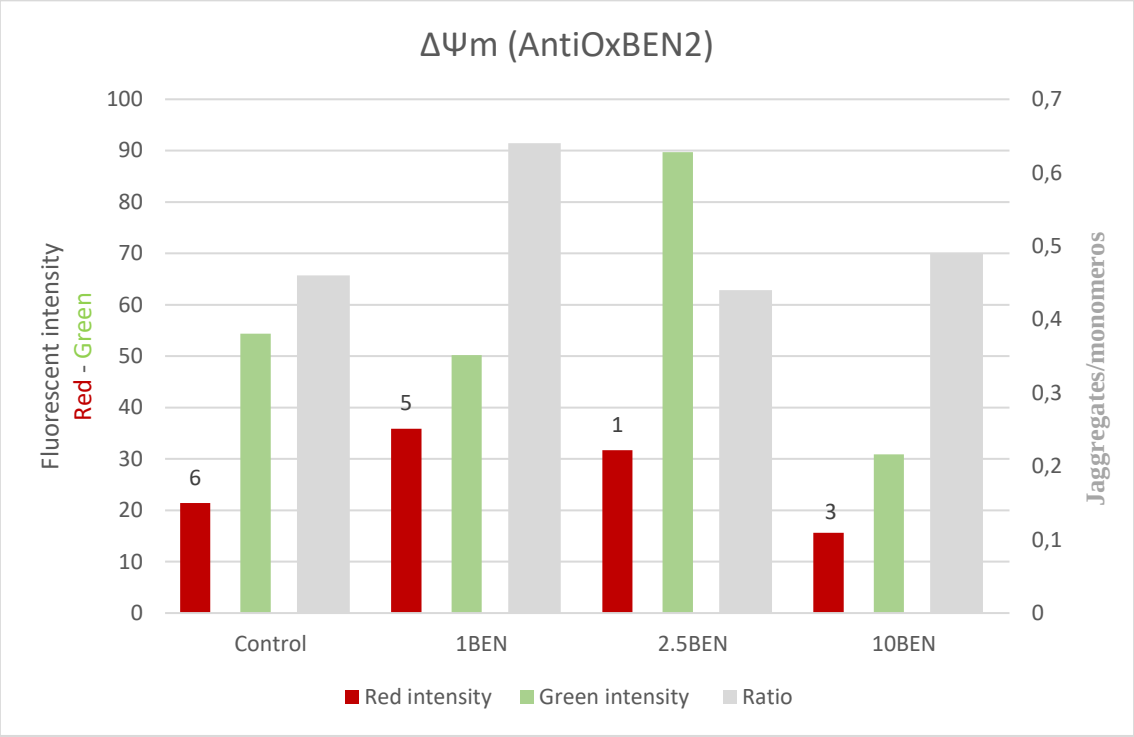

Figure 13 Assessment of mitochondrial membrane potential in embryos cultured in medium supplemented with AntiOxBEN2, using JC1dye. The red and green intensities were calculated as an average according to the embryo number available at day 9 of development in each group, Control, 1BEN, 2.5BEN, and 10BEN. Results of Ratio (Jaggregates / Jmonomeros) are presented in average of embryos in each group. Number above the Red intensity are the embryos assessed in each group.

Experiment 3

After the two previous experiments, the best concentration of the two antioxidants AntiOxCIN4 and AntiOxBEN2 were chosen based mainly on the analysis of embryonic quality. As mentioned above, embryo cryopreservation is enhancing worldwide. Therefore, embryo survival after vitrification and warming processes is of primordial importance to ART success. Our goal was to study embryo cryosurvival and developmental competence, comparing both antioxidants effects during embryo culture and after vitrification. In this experiment, the embryonic development and quality before and after the vitrification/warming process were analysed.

The embryo production data of the experiment 3 were depicted in Table 7. Similarly, to the previous experiments no significant differences were identified between groups.

Table 7 Effect of the best concentration of the two antioxidants (2.5 µM AntiOxCIN4 and 2.5 µM AntiOxBEN2) supplemented to the developmental medium on embryo production. The data is presented in total sum of 6 sessions; n, number of embryos and in percentage (%) as the mean of the calculations explained in the methodology ± the Standard Error Mean. D8 corresponded to eighth day of development.

GROUPS	PRESUMPTIVE ZYGOTES	CLEAVAGE		D8 EMBRYOS	
		N	%	N	%
CONTROL	331	245	77.3±3.6	31	12.7±2.6
2.5CIN	325	226	69.9±3.7	27	13.4±2.8
2.5BEN	310	222	72.1±3.2	35	17.8±3.3

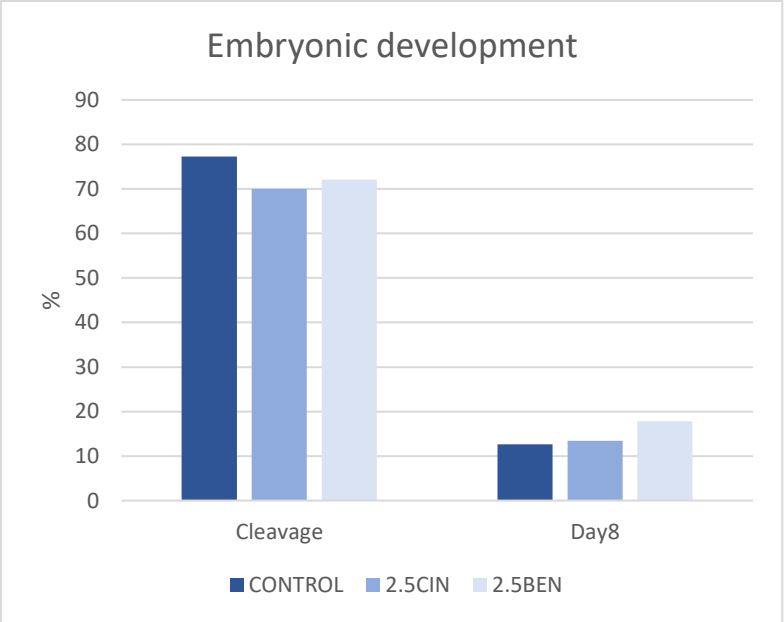

Figure 14 Effect of the best concentration of AntiOxCIN4 and AntiOxBEN2 (2.5µM CIN, 2.5µM BEN) supplemented to the developmental medium on production ratios at Day 2 (cleavage) and Day 8 (morulae and blastocysts).

A tendency (P=0.08) for an effect of antioxidants on embryo quality at day 8 of development, was identified in this experiment (Figure 15). The embryos supplemented with 2.5CIN had the highest percentage of Very Good/Good quality embryos (51.9%, P=0.04). Group 2.5BEN was the following group with a percentage of 42.9% (P=0.08) compared with the Control group (19.4%). In what concerns the other categories, Fair and Bad, the Control group had the biggest percentage, however without statistical significance.

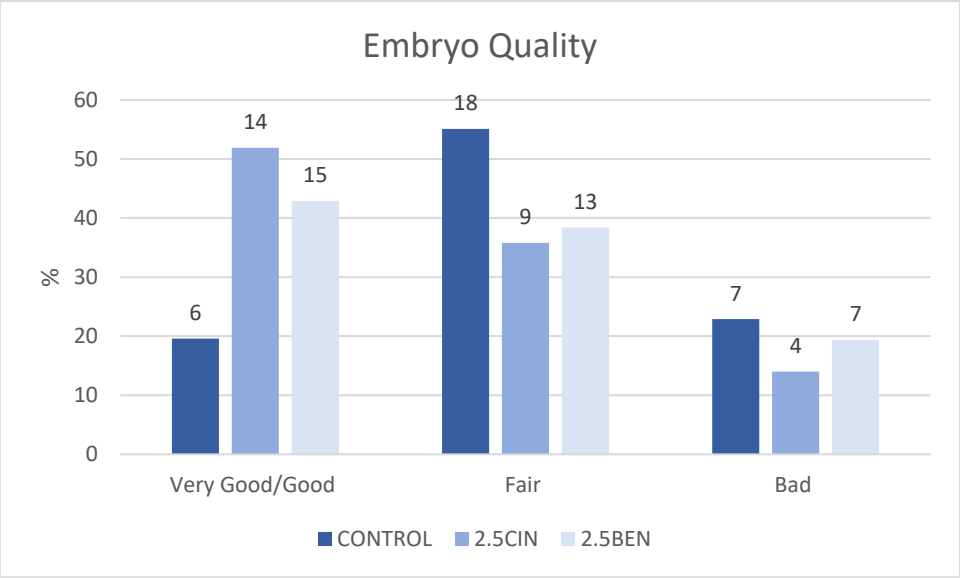

Figure 15 Effect of the best concentration of the two antioxidant (2.5 µM AntiOxCIN4 and 2.5 µM AntiOxBEN2) present in the developmental medium on the quality of produced embryos. The embryo quality was classified based on morphological criteria (Very Good/Good, Fair and Bad). Results presented in percentage and as total embryos in each group above bars.

Only embryos of very good/good and fair quality were vitrificated. Then to have a perspective of pregnancy success, it is important as well to examine the embryo quality after the vitrification/warming processes. At this point, the integrity of the embryo, its expansion immediately after warming, and after 24h of culture were evaluated (Figure 16).

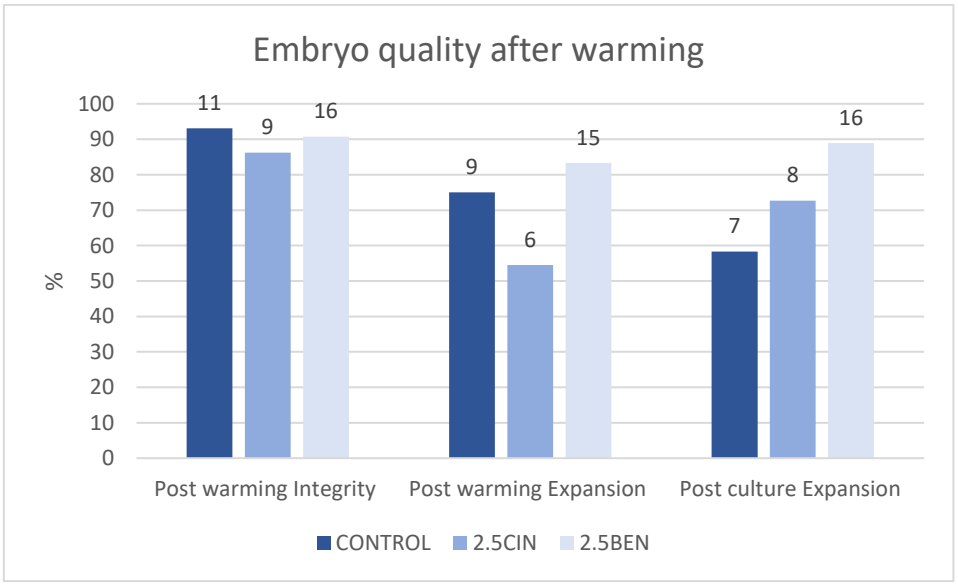

Figure 16 Effect of the best concentration of the two antioxidant 2.5 µM AntiOxCIN4 and 2.5 µM AntiOxBEN2 supplemented to the developmental medium on the quality of vitrified embryos after warming (Integrity and Expansion) and 24h of culture (Expansion in culture). The embryo quality is classified based on morphological criteria. Results presented in percentage and as total embryos in each group above bars.

Embryo integrity results were very similar among the three groups reaching near 90% of integrity (Figure 16). Concerning embryo expansion immediately after warming, no significant differences were identified between groups, although the 2.5CIN group had the lowest ratio. However, after 24 hours of culture more embryos belonging to the supplemented groups (2.5BEN: 16/18 and 2.5CIN: 8/11) were capable of expanding than in control group (7 /12)

Moreover, when embryo expansion was scored as follows: expansion: 3 points; semi-expansion: 2 points; and shrunken: 1 point; superior results were obtained after supplementation with 2.5BEN. In fact, when embryo expansion after culture was compared between groups (Figure 17) a significant difference (P=0.05) between the Control group and the 2.5 BEN group was identified. As represented in figure 17, the 2.5 BEN group achieved more expanded embryos (64.7%) and semi-expanded (29.4%) than the Control group (36.4% and 27.3%, respectively). The two groups with the supplementation of the antioxidants did not have a significant difference in terms of expanded embryos per group. Conversely the 2.5 CIN group had a trend (P=0.09) compared to the Control group.

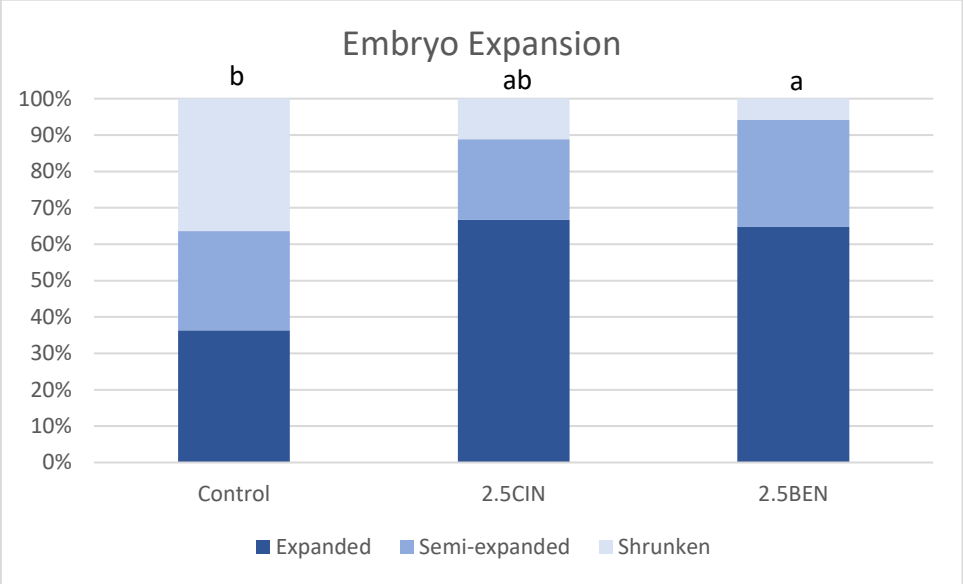

Figure 17 Effect of culture medium supplementation with antioxidants (Control, 2.5 CIN, 2.5BEN) on embryo expansion after 24h of vitrified-warming. The embryo expansion was classified based on morphological criteria. Results presented in percentage. Different letters indicate statistical difference between groups through the Mann-Whitney U Test.

Assessment of embryonic viable cells

As in the other two experiments, cell viability, ROS level, and $\Delta\Psi m$ were examined but in vitrified/warmed embryos.

In Table 8, it is possible to see the data from embryo total and death cells assessment and their viability rate. Similar results were obtained in all groups with a slight increase of viability in the presence of antioxidants, although without statistical significance.

Table 8 Assessment of total and viable cells after embryo culture with the two antioxidants (2.5 µM AntiOxCIN4 and AntiOxBEN2) and vitrified/warmed. It was calculated the total cell number with Hoechst dye, the number of dead cells through Pi, and estimated the percentage of Viable cells, after embryo vitrification and warming. Data are expressed as averages ± SD (Standard Deviation

GROUPS	N	TOTAL CELL (N)	DEATH CELL (N)	VIABLE CELL%
CONTROL	5	62.9±11.5	44.0±9.2	27.4±12.7
2.5CIN	3	62.0±12.4	40.7±10.6	34.7±12.7
2.5BEN	4	57.2±11.4	41.3±9.2	34.4±12.7

In this experiment, ROS levels were not significantly different between groups (Table 9) just 2.5 BEN presented a slightly lower intensity value compared with the two other groups.

Table 9 Assessment of ROS level in embryos cultured in medium supplemented *with* the two antioxidant 2.5 µM AntiOxCIN4 and 2.5 µM AntiOxBEN2, analysed using the CellRox dye. The CellRox intensity was calculated as an average according to the embryo number available after warming in each group. Data are expressed as averages.

GROUPS	N	CELLROX INTENSITY
CONTROL	4	19.0±1.9
2.5CIN	3	20.0±2.2
2.5BEN	4	16.5±1.9

In the assessment of the ΔΨm (Figure 18), 2.5BEN demonstrated the highest intensity of green dye and 2.5CIN had the highest Jc-1 ratio, although no statistically significant differences stood out.

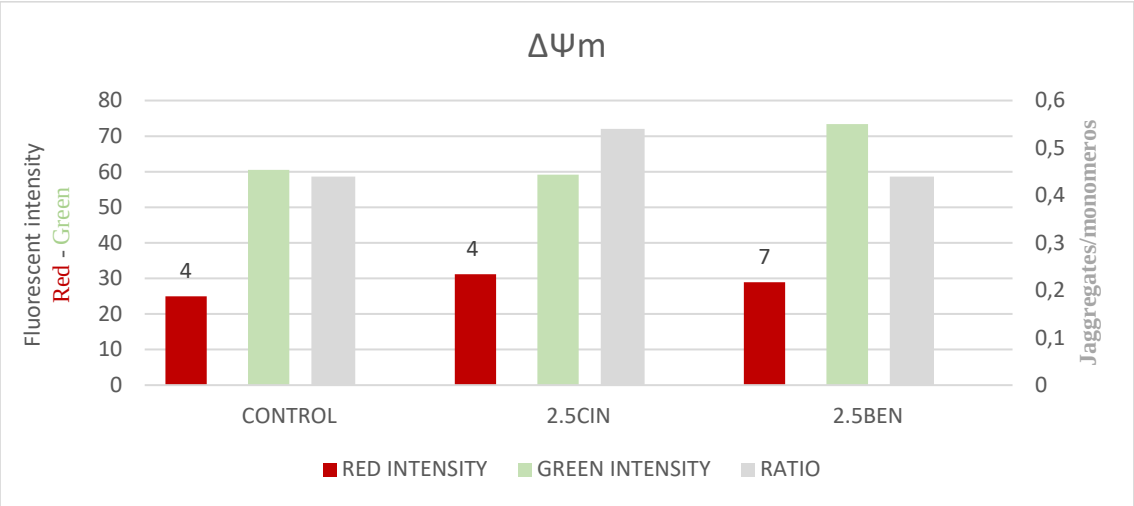

Figure 18 Assessment of the Mitochondrial Membrane Potential in embryos cultured in a medium supplemented *with* the two antioxidant (2.5 µM AntiOxCIN4 and AntiOxBEN2) and vitrified/warmed. The red and green intensities were calculated as an average according to the embryo number available at day 8 of development in each group, Control, 2.5CIN and 2.5BEN. Results of Ratio (Jaggregates / Jmonomeros) are presented in average of embryos in each group. Number above the Red Intensity bar are the embryos assessed in each group.

Discussion

The demand for ART is increasing worldwide both in humans and in animals. Since the first IVF baby, the search for ART in humans has increased tremendously over time. In the animal industry, these techniques are widely used to assure a specific breed or the safeguard of species/subspecies but also to increase the production of genetic superior animals. Embryo production and transfer is a worldwide technique used in thousands of laboratories. Although the quality of *in vitro* produced embryos has improved overtime, a lower quality persists when compared with the in vivo developed embryos. Oxidative stress has been pointed out as one of the major aetiologies for this discrepancy. However, an absence of successfully oxidative stress therapies, contributing to fix this gap between the *in vivo* and *in vitro* embryo's qualities, remain and are urgently needed (Duranthon & Chavatte-Palmer, 2018; Martin-Hidalgo et al., 2019; Kageyama et al., 2021). Therefore, the aim of this work was the study of the two novel antioxidants, AntiOxCIN4 and AntiOxBEN2, during embryo culture as a response to the oxidative stress problem. Our results, showed for the first time, that embryo supplementation with these antioxidants directed to mitochondria increased the quality and cryosurvival of produced blastocyst.

The two antioxidants, AntiOxCIN4 and AntiOxBEN2, were also successfully used by our team during the *in vitro* capacitation of spermatozoa, the fertilization (Santos, 2020) and the maturation of oocytes processes (Teixeira et al., 2020). Besides the supplementation on a different stage of development and culture medium, this work had the goal to study the best concentration of the two antioxidants (1 µM, 2.5 µM and 10 µM) and the influence of these drugs in the embryo recovery after vitrification-warming.

During our research, cleavage and blastocyst rates were firstly assessed in the first and second experiments to understand if the different media supplementation can produce more embryos than the control group. Several studies (Hosseini et al., 2009; Sun et al., 2015; Anjos et al., 2019; dos Santos et al., 2019) present an increased production rates when the culture medium was supplemented with other types of antioxidants. In our work, a tendency to an increase of the cleavage rates at the concentrations of 1CIN and 2.5CIN was also verified (Table 1 and Figure 4). These values suggest a better embryo development at those concentrations, which resulted also in fewer embryos with low quality in the groups 2.5CIN and 10CIN (Figure 7).
Concerning the embryos supplemented with AntiOxBEN2 (Table 5), no statistically relevant differences between concentrations were observed in the cleavage or blastocyst rates. These results seem to be in accordance with Torres et al., (2019) that revealed a possible independency of the two factors cleavage/blastocyst rates and quality. In fact, Torres et al. (2019) did not detect an increase in the cleavage or blastocyst rates despite achieving a raise in the quality of embryos. Our work followed the same path. The group 2.5BEN presented good results in the quality assay having a higher average of Very Good/Good embryos (Figure 11), which distinguished them from the rest of the groups. Numerous studies have demonstrated that embryo quality is one of the most trustworthy parameters with impact on pregnancy rates (Coleman et al., 1987; Putney et al., 1988; Erdem et al., 2020). Furthermore, higher quality embryos are correlated with fewer embryonic deaths and a better development and pregnancy rates (Erdem et al., 2020).

When the AntiOxCIN4 and AntiOxBEN2 were supplemented in other phases of the IVF techniques in the studies by Teixeira et al., 2020 and Santos, 2020, several beneficial effects were identified in the embryos as well as in the gametes. In spermatozoa, AntiOxBEN2 was used with

a concentration of 1µM and 10µM leading to an increased production rate but without interfering on embryo quality (Santos, 2020). Conversely, the supplementation of the antioxidants in the oocyte maturation medium with concentrations of 10µM, 20µM, 50µM and 100 µM of AntiOxCIN4 and AntiOxBEN2 had a dose dependent effect. The lowest concentrations of AntiOxBEN2 (10µM and 20µM) increased embryo quality and number of produced embryos, while the higher doses presented a toxic effect harming embryo development. The addiction of CIN had beneficial effects on the Transmembrane Electric Potential of the oocyte but did not have any advantages for the quality or number of produced embryos (Teixeira et al., 2020). These results strengthened that a dose dependent effect occurs and that lower concentrations of AntiOxCIN4 and AntiOxBEN2 maybe more beneficial for ART.

Comparing the embryo qualities obtained by Teixeira et al. (2020), Santos (2020) and our work, AntiOxBEN2 achieved better results when supplemented in the embryo culture medium reaching a difference of 35% more embryos of good quality. The supplementation with AntiOxCIN4 had similar values between the works.

To further study the higher embryo quality and identify the mechanism of action of antioxidants in embryo physiology, other methods were applied, such as the assessment of embryonic viable cells, ΔΨm and ROS levels. Corroborating the good quality results in 2.5BEN group, a tendency for a higher number of embryo total cells was also verified (Figure 12). According to several authors, higher number of total cells is correlated with a superior embryo quality and a higher survival rate after transfer (Iwasaki et al., 1990; Korhonen et al., 2010). The other assessments did not showed strong statistical differences among concentrations or antioxidants. However, Teixeira et al. (2021) demonstrated that AntiOxBEN2 and AntiOxCIN4 did not alter ΔΨm in primary human skin fibroblasts and decreased ROS levels. Further studies with higher number of embryos should be performed to understand how the antioxidants alter the mitochondria and the embryo. Similar problem has occurred in the direct method for the assessment of ROS, in all experiments. Unfortunately, the analysis did not achieve clear results, not only due to the low ratio of blastocysts allied to the multiple experiments and groups tested, but also because of all the constrains that the covid-19 pandemic brought us, taking from us weeks of work necessary to have more robust results. Clearly AntiOxCIN4 and AntiOxBEN2 have antioxidants properties that were already identified in different cells and models (Teixeira et a., 2017a, b 2018, 2020). Both antioxidants belong to a subclass of phenolic acids that have intrinsic capacities of *methoxylation, and an* ortho-catechol system that has the capacity to donate electrons or hydrogen to the free radicals. Due to these capacities, both demonstrated to have remarkable antioxidant and iron-chelating properties, preventing lipid peroxidation, and the capacity of modulate antioxidant-related gene express (Teixeira et al., 2017a,b).
Moreover, Teixeira et al. (2021) have suggested that both antioxidants could act as dose-dependently prooxidants being also described as pan-assay-interference compound (PAINS) or hermetic-like effect, which contributes to a lower level of toxicity, but also promotes health benefits and stimulates cellular defence responses (Teixeira et al., 2017a, b). In primary human skin fibroblasts cultures supplemented with AntiOxBEN2 and AntiOxCIN4, Teixeira et al. (2021), firstly identified an increase of ROS, which triggered the endogenous protection (upsurge of GSH and SOD levels), followed by a decrease of ROS. This response has restored the homeostatic circuits preventing cell death.
Based on the embryo quality scores, it was concluded that the best concentration of both AntiOxCIN4 and AntiOxBEN2 was 2.5µM. Therefore, these concentrations were used in the

third experiment. This last experiment had the purpose of comparing the embryo's survival after the vitrification-warming process, as well as comparing the two antioxidants added in the initial phases of embryonic development with each other, in the same experiment.

Since the first revival of a mammal cryopreserved organism in 1952 (Polge & Rowson, 1952) in *Bos taurus* spermatozoa cells, there has been several improvements and today embryo's cryopreservation has a survival rate of 78-100% (Rienzi et al., 2017). In this work, the integrity of post-warming embryos was close to 100% in all groups (Figure 16) and their expansion achieved values near to 90%. This indicates not only the good embryos quality but also the success of the vitrification-warming method. As proved before the antioxidant supplementation during embryo culture improved their quality. In the category of embryos with a Very Good /Good quality, the 2.5CIN and 2.5BEN obtained values above the Control Group (Figure 14). Our data also demonstrated that this improved quality was reflected in better cryosurvival rates, as expected due to the established correlation between quality and cryosurvival (Fidelis et al., 2020). After analysing the results of the recovery/expansion assays, the 2.5BEN group achieved the best results, attaining more expanded embryos (Figure 17) when compared to the other groups. The group 2.5CIN also showed a tendency to higher expansion rates after vitrification and post warming culture. According to Shear et al., (2020) this high percentage of expanded embryos is related with a superior quality and the highest likelihood of live birth.

The major problems associated with cryosurvival depend on cellular dehydration, due to the osmotic stress and damage in the cell membrane as well as the membrane of organelles due to the concentration and toxicity of cryoprotectants (Lane et al., 2002; Jin & Mazur, 2015; Marques et al., 2018). One of the organelles hardly damaged by the cryopreservation process is the mitochondria as well as its electronic transport system, consequently affecting the oxidative phosphorylation and the ROS production (Lane et al., 2002; Hosseini et al., 2009). Numerous studies confirm an increase of O_2^- levels in cryopreserved-warmed embryos and a modification of the normal values of the $\Delta\Psi m$ (reviewed in Len et al., 2019).

Comparing both antioxidants best concentrations, it seems that the AntiOxBEN2 demonstrated the best performance to increase the embryo quality as well as cryosurvival. Although the AntiOxCIN4 showed a higher antioxidant capacity than AntiOxBEN2 in other studies (Teixeira et al., 2017, a, b), in the present work it was not possible to verify differences of ROS levels or $\Delta\Psi m$. Moreover, a higher accumulation capacity of AntiOxBEN2 in the mitochondria, demonstrating to be a better target and a more stable antioxidant was also identified (Teixeira et al., 2017,b) that may be responsible for these important effects on embryo quality and cryosurvival improvement.

The supplementation of culture media with different antioxidants at different stages of *in vitro* embryo production has been tried by several authors to combat the rising of ROS during the process of cryopreservation-warming. Carrillo-González & Maldonado-Estrada (2020) and Zolini et al. (2019) supplemented the oocyte maturation medium and the embryo culture medium with L-carnitine and had opposite results. Carrillo-González & Maldonado-Estrada (2020) stated that the addition of 3.8 mM of L-carnitine during *in vitro* maturation, and of 1.5 mM later, during late embryo culture, had no effect on the embryo cryosurvival while Zolini et al. (2019) using a lower concentration (0.75mM) obtained good results improving embryo cryosurvival. The higher concentration used by Carrillo-González & Maldonado-Estrada (2020) although without apparent effect on embryo cryotolerance, improved the pregnant rate of transferred embryos.

Moreover, Held-Hoelker et al. (2017) supplemented L-carnitine only to the embryo culture medium and obtained an improvement of their cryosurvival as well. According to Li et al. (2021) different antioxidants or different concentrations of the same antioxidant can alter the obtained

results, but different stages of *in vitro* embryo production or methodologies also can change it. Hosseini et al. (2009) used a different type of molecule (β-mercaptoethanol) and achieved interesting results. They argued that the supplementation of antioxidants only in the embryo culture medium might be not enough to maintain the redox state of these embryos during the critical period of post-warming culture. Therefore, the addition of the antioxidants should be performed in the culture medium pre- and post-warming to maintain the embryo in a healthy redox stage.

AntiOxCIN4 and AntiOxBEN2 have already been demonstrated to be promising antioxidants, not only to the embryo but as well in other study models for the combat against the oxidative stress. As referred, in human skin fibroblasts, the two antioxidants reduced ROS through the prooxidant factor, not affecting the ΔΨm (Teixeira et al., 2021) while in the maturation of oocytes AntiOxCIN4 improved ΔΨm, contrasting to AntiOxBEN2 that did not (Teixeira et al., 2020). Moreover, in skin fibroblasts it was showed the capacity to trigger an endogenous protective action, increasing the levels of GSH and SOD protein levels. This side effect was responsible for the decrease of ROS. However, AntiOxCIN4 and AntiOxBEN2 had different impacts on the fibroblasts. AntiOxBEN2 revealed to be an antagonist of H_2O_2, being much more effective than AntiOxCIN4, as it was hypothesized in our work. The two antioxidants have different expressions of HMOX1, NQQ1, and NFR2, three modulators responsible for the cellular antioxidant response. AntiOxBEN2 induces a higher expression of these genes, and this factor might be linked with the effectiveness of the antioxidant in combating the cells death (Teixeira et al., 2021). The obtained antioxidants response was considered therapeutically valuable for neurodegenerative diseases as well. In one study where Neuroblastoma model cells were used, the results showed that the antioxidants prevented neurodegeneration. AntiOxCIN4 and AntiOxBEN2 did not alter the cells metabolic activity but, once again, increased the ROS level, operating as a prooxidant, stimulating an endogenous pathway, therefore reducing oxidative stress, and avoiding free iron as well (Benfeito et al., 2019).

Although both antioxidants showed the capacity to increase cellular GSH and mitochondrial SOD protein levels as showed in other studies, it is necessary to demonstrate the same capability in embryos, as well as the capacity to not alter the ΔΨm and confirm a decrease in the levels of ROS. Also, more studies are needed to confirm our preliminary results, not only to be confirmed but also to understand how the antioxidants can alter the antioxidant-related gene expression in the embryos. Another interesting point of view will be to study the simultaneously antioxidant supplementation in other phases of IVF. All the phases of IVF suffer an increase of ROS. Some authors defend that oocyte quality is the most important component to determinate developmental rates, being an important phase to the addition of antioxidants (Krisher, 2004; Ferré et al., 2020). Spermatozoa on other hand do not have the capacity to repair the damage induced by the oxidative stress (Agarwal et al., 2005). Therefore, the addition of the antioxidants in the capacitation and fertilization media could be efficient in the final result (Santos, 2020). It could also be more beneficial to use cocktails of antioxidants in different phases of development (Bilska & Włodek, 2005; Truong et al., 2016), because the isolation of the antioxidants showed poorer results or even deleterious effects depending on the developmental stage (Truong et al., 2016). As an example, the supplementation of EDTA in the early stage of development revealed good results but in later stages showed negative effects for the embryo (Guérin et al., 2001).

Recently, other mitochondrial directed drugs have been used as therapies for oxidative stress, such as SNO in vascular oxidative stress (Shi & Qiu, 2020), and SkQ1 in placental oxidative stress (Hebert & Myatt, 2021). These molecules have the same nanocarrier, TPP, of the antioxidants studied in this work and achieved good results in decreasing ROS production. In

addition, MitoQ is one of the most studied antioxidants for the reduction of ROS because of its good results (Teixeira et al., 2017; Teixeira et al., 2017, b). MitoQ has increased embryo quality and upregulated genes, which are valuable for endogenous protection (Marei et al., 2019). Presently, this antioxidant is already being used in the cosmetics industry (Veloso, 2019). It obtained good results in the decrease of ROS below the critical threshold that induces apoptosis, consequently decreasing the oxidative stress (Marei et al., 2019). Despite the good results, Kageyama et al., 2021, presented an antioxidant that also had TPP, but more efficient than the MitoQ. These authors added MitoQ and MitoT, the new antioxidant, to the culture medium, and on the first day of development the embryos produced a low level of ROS and the ones supplemented with MitoT also exhibited a low mitochondrial DNA copy, a condition associated with a higher implementation potential (Fragouli et al., 2015; Kageyama et al., 2021). The data did not demonstrate any modification in the total number of cells and the NRF2 was not upregulated, unlike the work done by Teixeira et al. (2021). This variation is due to the differences in the performances of each antioxidant, even though all four of them have the same strategy to be accumulated in the mitochondria, via a lipophilic cation. The AntiOxBEN2, as well as the AntiOxCIN4, worked as antioxidants our prooxidants, while MitoQ and MitoT performed as simple antioxidants. AntiOxCIN4 and AntiOxBEN2 already demonstrated a higher antioxidant capacity than MitoQ (Teixeira et al., 2017; b). A study comparing the effects of AntiOxCIN4, AntiOxBEN2, and MitoT in ART will be of high interest to demonstrate which of the mentioned antioxidants could have a higher antioxidant activity as a better target to the mitochondria.

In conclusion, an efficient therapy to mitigate the oxidative stress with mitochondria as a target and therefore avoiding toxicity for embryo physiology and promoting its successful development, is currently needed. Now more than ever this therapy is required to face the negative impact that the industrial world and climate changes have on gametes and pregnancies rates (Keurst et al., 2016; Rahman et al., 2018; Cavallari et al., 2019; Fonseca et al, 2020). The results obtained in this study were very promissory since our aim of increasing embryo quality and cryosurvival, was reached. The best concentration of the two antioxidants, 2.5µM of AntiOxCIN4 or AntiOxBEN2, when supplemented during the initial phases of embryonic development improved the quality and cryotolerance of produced embryos, where AntiOxBEN2 stood out. This mithocondriotropic antioxidant has increased the number of very good/good quality embryos and their total cell number and allowed a better recovery after vitrification-warming.

References:

Abedal-Majed, M. A., & Cupp, A. S. (2019). Livestock animals to study infertility in women. *Animal Frontiers*, *9*(3), 28–33. https://doi.org/10.1093/af/vfz017

Adams, G. P., & Pierson, R. A. (1995). Bovine model for study of ovarian follicular dynamics in humans. *Theriogenology*, *43*(1), 113–120. https://doi.org/10.1016/0093-691X(94)00015-M

Adams, G. P., Singh, J., & Baerwald, A. R. (2012). Large animal models for the study of ovarian follicular dynamics in women. *Theriogenology*, *78*(8), 1733–1748. https://doi.org/10.1016/j.theriogenology.2012.04.010

Agarwal, A., Gupta, S., & Sharma, R. (2005). Oxidative stress and its implications in female infertility - A clinician's perspective. *Reproductive BioMedicine Online*, *11*(5), 641–650. https://doi.org/10.1016/S1472-6483(10)61174-1

Agarwal, A., Said, T. M., Bedaiwy, M. A., Banerjee, J., & Alvarez, J. G. (2006). Oxidative stress in an assisted reproductive techniques setting. *Fertility and Sterility*, *86*(3), 503–512. https://doi.org/10.1016/j.fertnstert.2006.02.088

Anjos, J. C., Aguiar, F. L. N., Sá, N. A. R., Souza, J. F., Cibin, F. W. S., Alves, B. G., Santos, R. R., & Figueiredo, J. R. (2019). Anethole improves blastocysts rates together with antioxidant capacity when added during bovine embryo culture rather than in the in vitro maturation medium. *Zygote*, 16–19. https://doi.org/10.1017/S0967199419000443

Baruselli, P., Vieira, L., Ferreira, R., Gimenes, L., Martins, C., Sá Filho, M., & Bó, G. (2015). Updates on embryo production strategies. *Animal Reproduction*, *12*(3), 375–382.

Bassols, A., Costa, C., Eckersall, P. D., Osada, J., Sabrià, J., & Tibau, J. (2014). The pig as an animal model for human pathologies: A proteomics perspective. *Proteomics - Clinical Applications*, *8*(10), 715–731. https://doi.org/10.1002/prca.201300099

Bedoschi, G., Roque, M., & Esteves, S. C. (2020). ICSI and Male Infertility: Consequences to Offspring. *Male Infertility. Springer, Cham.*, 765–775. https://doi.org/https://doi.org/10.1007/978-3-030-32300-4_61

Benfeito, S., Oliveira, C., Fernandes, C., Cagide, F., Teixeira, J., Amorim, R., Garrido, J., Martins, C., Sarmento, B., Silva, R., Remião, F., Uriarte, E., Oliveira, P. J., & Borges, F. (2019). Fine-tuning the neuroprotective and blood-brain barrier permeability profile of multi-target agents designed to prevent progressive mitochondrial dysfunction. *European Journal of Medicinal Chemistry*, *167*, 525–545. https://doi.org/10.1016/j.ejmech.2019.01.055

Bilska, A., & Włodek, L. (2005). Lipoic acid - The drug of the future? *Pharmacological Reports*, *57*(5), 570–577.

Brandt, J. S., Cruz Ithier, M. A., Rosen, T., & Ashkinadze, E. (2019). Advanced paternal age, infertility, and reproductive risks: A review of the literature. *Prenatal Diagnosis*, *39*(2), 81–87. https://doi.org/10.1002/pd.5402

Buettner, G. R. (1993). The Pecking Order of Free Radicals and Antioxidants: Lipid Peroxidation, α-Tocopherol, and Ascorbate. In *Archives of Biochemistry and Biophysics* (Vol. 300, Issue 2, pp. 535–543). https://doi.org/10.1006/abbi.1993.1074

Burton, G. J., Hempstock, J., & Jauniaux, E. (2003). Oxygen, early embryonic metabolism and free radical-mediated embryopathies. *Reproductive BioMedicine Online*, *6*(1), 84–96. https://doi.org/10.1016/S1472-6483(10)62060-3

Carrillo-González, D. F., & Maldonado-Estrada, J. G. (2020). L-carnitine supplementation in culture media improves the pregnancy rate of in vitro produced embryos with sexed semen from Bos taurus indicus cows. *Tropical Animal Health and Production*, *52*(5), 2559–2565. https://doi.org/10.1007/s11250-020-02281-y

Cavallari, F. D. C., Leal, C. L. V., Zvi, R., & Hansen, P. J. (2019). Effects of melatonin on production of reactive oxygen species and developmental competence of bovine oocytes exposed to heat shock and oxidative stress during in vitro maturation. *Zygote*, *27*(3), 180–186. https://doi.org/10.1017/S0967199419000236

Chavarro J.E., Rich-Edwards J.W., Rosner B.A., W. W. C. (2007). *Diet and Lifestyle in the Prevention of Ovulatory journals.lww.com*. *110*(5), 1050–1058. http://journals.lww.com/greenjournal/Fulltext/2007/11000/Diet_and_Lifestyle_in_the_Prevention_of_Ovulatory.17.aspx

Choi, J., Baek, J., Kweon, D., Ko, K. S., & Yoon, H. (2020). Rapid determination of carbapenem resistance by low-cost colorimetric methods: Propidium Iodide and alamar blue staining. *Journal of Microbiology*, *58*(5), 415–421. https://doi.org/10.1007/s12275-020-9549-x

Coleman, D. A., Dailey, R. A., Leffel, R. E., & Baker, R. D. (1987). Estrous Synchronization and Establishment of Pregnancy in Bovine Embryo Transfer Recipients. *Journal of Dairy Science*, *70*(4), 858–866. https://doi.org/10.3168/jds.S0022-0302(87)80084-X

De Munck, N., Janssens, R., Segers, I., Tournaye, H., Van De Velde, H., & Verheyen, G. (2019). Influence of ultra-low oxygen (2%) tension on in-vitro human embryo development. *Human Reproduction*, *34*(2), 228–234. https://doi.org/10.1093/humrep/dey370

Divyashree, S., & Yajurvedi, H. N. (2018). Chronic stress effects and their reversibility on the Fallopian tubes and uterus in rats. *Reproduction, Fertility and Development*, *30*(2), 380–390. https://doi.org/10.1071/RD17082

Dominguez, A. A., & Reijo Pera, R. A. (2013). Infertility. *Brenner's Encyclopedia of Genetics: Second Edition*, *2*, 71–74. https://doi.org/10.1016/B978-0-12-374984-0.00793-2

dos Santos, E. C., Varchetta, R., de Lima, C. B., Ispada, J., Martinho, H. S., Fontes, P. K., Nogueira, M. F. G., Gasparrini, B., & Milazzotto, M. P. (2019). The effects of crocetin supplementation on the blastocyst outcome, transcriptomic and metabolic profile of in vitro produced bovine embryos. *Theriogenology*, *123*, 30–36. https://doi.org/10.1016/j.theriogenology.2018.08.010

du Plessis, S. S., Makker, K., Desai, N. R., & Agarwal, A. (2008). Impact of oxidative stress on stress on IVF. *Differentiation*, *99*, 21–27. https://doi.org/10.1016/j.diff.2017.12.002

Duranthon, V., & Chavatte-Palmer, P. (2018). Long term effects of ART: What do animals tell us? *Molecular Reproduction and Development*, *85*(4), 348–368. https://doi.org/10.1002/mrd.22970

Erdem, H., Karasahin, T., Alkan, H., Dursun, S., Satilmis, F., & Guler, M. (2020). Effect of embryo quality and developmental stages on pregnancy rate during fresh embryo transfer

in beef heifers. *Tropical Animal Health and Production*, *52*(5), 2541–2547. https://doi.org/10.1007/s11250-020-02287-6

Ernster, L., Nordenbrand, K., & Forsmark, P. (1992). S-19-4 The Mode of Action of Lipid-Soluble Antioxidants in Biological Membranes.Relationship between the Effects of Ubiquinol and Vitamin E as Inhibitors of Lipid Peroxidation in Submitochondrial Particles. *Journal of Nutritional Science and Vitaminology*, *38*, 548–551. https://doi.org/10.3177/jnsv.38.Special_548

Esteves, S. C., Roque, M., Bedoschi, G., Haahr, T., & Humaidan, P. (2018). Intracytoplasmic sperm injection for male infertility and consequences for offspring. *Nature Reviews Urology*, *15*(9), 535–562. https://doi.org/10.1038/s41585-018-0051-8

F, B. (2014). Infertility. *Elsevier Inc*, 2019. https://doi.org/10.1016/B978-0-12-801238-3.00268-3

Fair, T., Lonergan, P., Dinnyes, A., Cottell, D. C., Hyttel, P., Ward, F. A., & Boland, M. P. (2001). Ultrastructure of bovine blastocysts following cryopreservation: Effect of method of blastocyst production. *Molecular Reproduction and Development*, *58*(2), 186–195. https://doi.org/10.1002/1098-2795(200102)58:2<186::AID-MRD8>3.0.CO;2-N

Ferlin, A., & Foresta, C. (2020). Infertility: Practical Clinical Issues for Routine Investigation of the Male Partner. *Journal of Clinical Medicine*, *9*(6), 1644. https://doi.org/10.3390/jcm9061644

Ferré, L. B., Kjelland, M. E., Strøbech, L. B., Hyttel, P., Mermillod, P., & Ross, P. J. (2020). Review: Recent advances in bovine in vitro embryo production: reproductive biotechnology history and methods. *Animal*, *14*(5), 991–1004. https://doi.org/10.1017/S1751731119002775

Fidelis, A. A. G., de Oliveira Fernandes, G., Melo, F. R., de Oliveira Leme, L., Adona, P. R., Kawamoto, T. S., & Dode, M. A. N. (2020). Ethanolic extract of dried leaves from the cerrado biome increases the cryotolerance of bovine embryos produced in vitro. *Oxidative Medicine and Cellular Longevity*, *2020*. https://doi.org/10.1155/2020/6046013

Figueroa-Espinoza, M. C., & Villeneuve, P. (2005). Phenolic enzymatic lipophilization. *Journal of Agricultural and Food Chemistry*, *53*(8), 2779–2787. https://doi.org/10.1021/jf0484273

Fischer, B., & Bavister, B. D. (1993). Oxygen tension in the oviduct and uterus of rhesus monkeys, hamsters and rabbits. *Journal of Reproduction and Fertility*, *99*(2), 673–679. https://doi.org/10.1530/jrf.0.0990673

Fonseca, É. (2020). *Anti-Aging Effect of Urolithin A on Bovine Oocytes*. Universidade de Coimbra.

Fonseca, E., Mesquita, P., Marques, C. C., Baptista, M. C., Pimenta, J., Matos, J. E., Soveral, G., & Pereira, R. M. L. N. (2020). Modulation of P2Y2 receptors in bovine cumulus oocyte complexes: effects on intracellular calcium, zona hardening and developmental competence. *Purinergic Signalling*, *16*(1), 85–96. https://doi.org/10.1007/s11302-020-09690-6

Fragouli, E., Spath, K., Alfarawati, S., Kaper, F., Craig, A., Michel, C. E., Kokocinski, F., Cohen, J., Munne, S., & Wells, D. (2015). Altered Levels of Mitochondrial DNA Are Associated with Female Age, Aneuploidy, and Provide an Independent Measure of Embryonic Implantation Potential. *PLoS Genetics*, *11*(6), 1–18.

https://doi.org/10.1371/journal.pgen.1005241

Gameiro, S. (2016). Infertility. *Encyclopedia of Mental Health: Second Edition*, *2*, 375–383. https://doi.org/10.1016/B978-0-12-397045-9.00159-2

Gaskins, A. J., & Chavarro, J. E. (2018). Diet and fertility: a review. *American Journal of Obstetrics and Gynecology*, *218*(4), 379–389. https://doi.org/10.1016/j.ajog.2017.08.010

Glujovsky, D., Blake, D., Bardach, A., & Farquhar, C. (2012). Cleavage stage versus blastocyst stage embryo transfer in assisted reproductive technology. *Cochrane Database of Systematic Reviews*, *2013*(6). https://doi.org/10.1002/14651858.CD002118.pub5

Glujovsky, D., Farquhar, C., Quinteiro Retamar, A. M., Alvarez Sedo, C. R., & Blake, D. (2016). Cleavage stage versus blastocyst stage embryo transfer in assisted reproductive technology (Review). *Cochrane Database of Systematic Reviews*, *2016*(6). https://doi.org/10.1002/14651858.CD002118.pub5

Grimm, A., & Eckert, A. (2017). Brain aging and neurodegeneration: from a mitochondrial point of view. *Journal of Neurochemistry*, *143*(4), 418–431. https://doi.org/10.1111/jnc.14037

Guérin, P., El Mouatassim, S., & Ménézo, Y. (2001). Oxidative stress and protection against reactive oxygen species in the pre-implantation embryo and its surroundings. *Human Reproduction Update*, *7*(2), 175–189. https://doi.org/10.1093/humupd/7.2.175

Guillette, L. J., & Edwards, T. M. (2005). Is nitrate an ecologically relevant endocrine disruptor in vertebrates? *Integrative and Comparative Biology*, *45*(1), 19–27. https://doi.org/10.1093/icb/45.1.19

Guzick, D. S., & Swan, S. (2006). The decline of infertility: apparent or real? *Fertility and Sterility*, *86*(3), 524–526. https://doi.org/10.1016/j.fertnstert.2006.05.027

Guzman-Villanueva, D., & Weissig, V. (2015). Mitochondria-Targeted Agents: Mitochondriotropics, Mitochondriotoxics, and Mitocans. *Handbook of Experimental Pharmacology*, *January*, 251–263. https://doi.org/10.1007/164

Guzman-Villanueva, D., & Weissig, V. (2016). Delivery of Biologically Active Molecules to Mitochondria. *Mitochondrial Mechanisms of Degeneration and Repair in Parkinson's Disease*, *B*, 1–275. https://doi.org/10.1007/978-3-319-42139-1

Hansen, P. J. (2020). Implications of Assisted Reproductive Technologies for Pregnancy Outcomes in Mammals. *Annual Review of Animal Biosciences*, *8*, 395–413. https://doi.org/10.1146/annurev-animal-021419-084010

He, C., Wang, J., Zhang, Z., Yang, M., Li, Y., Tian, X., Ma, T., Tao, J., Zhu, K., Song, Y., Ji, P., & Liu, G. (2016). Mitochondria synthesize melatonin to ameliorate its function and improve mice oocyte's quality under in vitro conditions. *International Journal of Molecular Sciences*, *17*(6), 1–16. https://doi.org/10.3390/ijms17060939

Hebert, J. F., & Myatt, L. (2021). Placental mitochondrial dysfunction with metabolic diseases: Therapeutic approaches. *Biochimica et Biophysica Acta - Molecular Basis of Disease*, *1867*(1), 165967. https://doi.org/10.1016/j.bbadis.2020.165967

Held-Hoelker, E., Klein, S. L., Rings, F., Salilew-Wondim, D., Zidane, M., Neuhoff, C., Tesfaye, D., Schellander, K., & Hoelker, M. (2017). Cryosurvival of in vitro produced

bovine embryos supplemented with L-Carnitine and concurrent reduction of fatty acids. *Theriogenology*, *96*, 145–152. https://doi.org/10.1016/j.theriogenology.2017.03.014

Hosseini, S. M., Forouzanfar, M., Hajian, M., Asgari, V., Abedi, P., Hosseini, L., Ostadhosseini, S., Moulavi, F., Safahani Langrroodi, M., Sadeghi, H., Bahramian, H., Eghbalsaied, S., & Nasr-Esfahani, M. H. (2009). Antioxidant supplementation of culture medium during embryo development and/or after vitrification-warming; Which is the most important? *Journal of Assisted Reproduction and Genetics*, *26*(6), 355–364. https://doi.org/10.1007/s10815-009-9317-7

Igarashi, H., Takahashi, T., Takahashi, E., Tezuka, N., Nakahara, K., Takahashi, K., & Kurachi, H. (2005). Aged mouse oocytes fail to readjust intracellular adenosine triphosphates at fertilization. *Biology of Reproduction*, *72*(5), 1256–1261. https://doi.org/10.1095/biolreprod.104.034926

Inhorn, M. C., & Patrizio, P. (2014). Infertility around the globe: New thinking on gender, reproductive technologies and global movements in the 21st century. *Human Reproduction Update*, *21*(4), 411–426. https://doi.org/10.1093/humupd/dmv016

Iwasaki, S., Yoshiba, N., Ushijima, H., Watanabe, S., & Nakahara, T. (1990). Morphology and proportion of inner cell mass of bovine blastocysts fertilized in vitro and in vivo. *Journal of Reproduction and Fertility*, *90*(1), 279–284. https://doi.org/10.1530/jrf.0.0900279

Jin, B., & Mazur, P. (2015). High survival of mouse oocytes/embryos after vitrification without permeating cryoprotectants followed by ultra-rapid warming with an IR laser pulse. *Scientific Reports*, *5*, 1–6. https://doi.org/10.1038/srep09271

Kageyama, M., Ito, J., Shirasuna, K., Kuwayama, T., & Iwata, H. (2021). Mitochondrial reactive oxygen species regulate mitochondrial biogenesis in porcine embryo. *Journal of Reproduction and Development*.

Kaiser, J. (2016). Endocrine Disrupters Trigger Fertility Problems in Multiple Generations. *Science*, *308*(5727), 1391–1392.

Kausar, S., Wang, F., & Cui, H. (2018). The Role of Mitochondria in Reactive Oxygen Species Generation and Its Implications for Neurodegenerative Diseases. *Cells*, *7*(12), 274. https://doi.org/10.3390/cells7120274

Kiesswetter, M., Marsoner, H., Luehwink, A., Fistarol, M., Mahlknecht, A., & Duschek, S. (2020). Impairments in life satisfaction in infertility: Associations with perceived stress, affectivity, partnership quality, social support and the desire to have a child. *Behavioral Medicine*, *46*(2), 130–141. https://doi.org/10.1080/08964289.2018.1564897

Kikuzaki, H., Hisamoto, M., Hirose, K., Akiyama, K., & Taniguchi, H. (2002). Antioxidant properties of ferulic acid and its related compounds. *Journal of Agricultural and Food Chemistry*, *50*(7), 2161–2168. https://doi.org/10.1021/jf011348w

Kishida, K. T., & Klann, E. (2007). Sources and targets of reactive oxygen species in synaptic plasticity and memory. *Antioxidants and Redox Signaling*, *9*(2), 233–244. https://doi.org/10.1089/ars.2007.9.233

Korhonen, K., Kananen, K., Ketoja, E., Matomäki, J., Halmekytö, M., & Peippo, J. (2010). Effects of serum-free in vitro maturation of bovine oocytes on subsequent embryo development and cell allocation in two developmental stages of day 7 blastocysts. *Reproduction in Domestic Animals*, *45*(1), 42–49. https://doi.org/10.1111/j.1439-

0531.2008.01203.x

Krisher, R. L. (2004). The effect of oocyte quality on development. *Journal of Animal Science*, *82 E-Suppl*, 14–23. https://doi.org/10.2527/2004.8213_supplE14x

Lämmle, C. A., Varady, A., Müller, T. G., Sturtzel, C., Riepl, M., Mathes, B., Eichhorst, J., Sporbert, A., Lehmann, M., Kräusslich, H. G., Distel, M., & Broichhagen, J. (2021). Photocaged Hoechst Enables Subnuclear Visualization and Cell Selective Staining of DNA in vivo. *ChemBioChem*, *22*(3), 548–556. https://doi.org/10.1002/cbic.202000465

Lane, M., Maybach, J. M., & Gardner, D. K. (2002). Addition of ascorbate during cryopreservation stimulates subsequent embryo development. *Human Reproduction*, *17*(10), 2686–2693. https://doi.org/10.1093/humrep/17.10.2686

Lapa, M., Marques, C. C., Alves, S. P., Vasques, M. I., Baptista, M. C., Carvalhais, I., Silva Pereira, M., Horta, A. E. M., Bessa, R. J. P., & Pereira, R. M. (2011). Effect of trans-10 cis-12 conjugated linoleic acid on Bovine Oocyte Competence and Fatty Acid Composition. *Reproduction in Domestic Animals*, *46*(5), 904–910. https://doi.org/10.1111/j.1439-0531.2011.01762.x

Laverge, H., De Sutter, P., Van Der Elst, J., & Dhont, M. (2001). A prospective, randomized study comparing day 2 and day 3 embryo transfer in human IVF. *Human Reproduction*, *16*(3), 476–480. https://doi.org/10.1093/humrep/16.3.476

Leibfried, L., & First, N. L. (1979). Characterization of bovine follicular oocytes and their ability to mature in vitro. *Journal of Animal Science*, *48*(1), 76–86. https://doi.org/10.2527/jas1979.48176x

Leite, R. F., Annes, K., Ispada, J., de Lima, C. B., dos Santos, É. C., Fontes, P. K., Gouveia Nogueira, M. F., & Milazzotto, M. P. (2018). Corrigendum to "Oxidative stress alters the profile of transcription factors related to early development on in vitro produced embryos" (Oxidative Medicine and Cellular Longevity (2017) 2017 (1502489) DOI: 10.1155/2017/1502489). *Oxidative Medicine and Cellular Longevity*, *2018*(Ivc). https://doi.org/10.1155/2018/6730857

Len, J. S., Koh, W. S. D., & Tan, S. X. (2019). The roles of reactive oxygen species and antioxidants in cryopreservation. *Bioscience Reports*, *39*(8). https://doi.org/10.1042/BSR20191601

Li, J., Liu, L., Weng, J., Yin, T. lang, Yang, J., & Feng, H. L. (2021). Biological roles of l-carnitine in oocyte and early embryo development. *Molecular Reproduction and Development*, *88*(10), 673–685. https://doi.org/10.1002/mrd.23542

Li, X., Mu, Y., Elshewy, N., Ding, D., Zou, H., Chen, B., Chen, C., Wei, Z., Cao, Y., Zhou, P., & Zhang, Z. (2021). Comparison of IVF and IVM outcomes in the same patient treated with a modified IVM protocol along with an oocytes-maturing system containing melatonin: A pilot study. *Life Sciences*, *264*(October 2020), 118706. https://doi.org/10.1016/j.lfs.2020.118706

Liu, Y., Han, M., Li, X., Wang, H., Ma, M., Zhang, S., Guo, Y., Wang, S., Wang, Y., Duan, N., Xu, B., Yin, J., & Yao, Y. (2017). Age-related changes in the mitochondria of human mural granulosa cells. *Human Reproduction*, *32*(12), 2465–2473. https://doi.org/10.1093/humrep/dex309

Luoma, J. (2005). Environmental Chemicals and Fertility Challenged Conceptions : *Biography*

An Interdisciplinary Quarterly.

Luvoni, G. C., Keskintepe, L., & Brackett, B. G. (1996). Improvement in bovine embryo production in vitro by glutathione-containing culture media. *Molecular Reproduction and Development*, *43*(4), 437–443. https://doi.org/10.1002/(SICI)1098-2795(199604)43:4<437::AID-MRD5>3.0.CO;2-Q

Magli, M. C., Van Den Abbeel, E., Lundin, K., Royere, D., Van Der Elst, J., & Gianaroli, L. (2008). Revised guidelines for good practice in IVF laboratories. *Human Reproduction*, *23*(6), 1253–1262. https://doi.org/10.1093/humrep/den068

Mailloux, R. J., & Treberg, J. R. (2016). Protein S-glutathionlyation links energy metabolism to redox signaling in mitochondria. *Redox Biology*, *8*, 110–118. https://doi.org/10.1016/j.redox.2015.12.010

Malhi, P. S., Adams, G. P., Mapletoft, R. J., & Singh, J. (2007). Oocyte developmental competence in a bovine model of reproductive aging. *Reproduction*, *134*(2), 233–239. https://doi.org/10.1530/REP-07-0021

Marei, W. F. A., Van Den Bosch, L., Pintelon, I., Mohey-Elsaeed, O., Bols, P. E. J., & Leroy, J. L. M. R. (2019). Mitochondria-targeted therapy rescues development and quality of embryos derived from oocytes matured under oxidative stress conditions: A bovine in vitro model. *Human Reproduction*, *34*(10), 1984–1998. https://doi.org/10.1093/humrep/dez161

Marín-García, J. (2012). *Targeting the Mitochondria in Cardiovascular Diseases*.

Marín-García, J., Akhmedov, A., Rybin, V., & Moe, G. W. (2013). Mitochondria and their role in cardiovascular disease. *Mitochondria and Their Role in Cardiovascular Disease*, *9781461445*, 1–500. https://doi.org/10.1007/978-1-4614-4599-9

Marques, C. C., Santos-Silva, C., Rodrigues, C., Matos, J. E., Moura, T., Baptista, M. C., Horta, A. E. M., Bessa, R. J. B., Alves, S. P., Soveral, G., & Pereira, R. M. L. N. (2018). Bovine oocyte membrane permeability and cryosurvival: Effects of different cryoprotectants and calcium in the vitrification media. *Cryobiology*, *81*(November 2017), 4–11. https://doi.org/10.1016/j.cryobiol.2018.03.003

Martin-Hidalgo, D., Bragado, M. J., Batista, A. R., Oliveira, P. F., & Alves, M. G. (2019). Antioxidants and male fertility: From molecular studies to clinical evidence. *Antioxidants*, *8*(4). https://doi.org/10.3390/antiox8040089

Martino, N. A., Ariu, F., Bebbere, D., Uranio, M. F., Chirico, A., Marzano, G., Sardanelli, A. M., Cardinali, A., Minervini, F., Bogliolo, L., & Dell'Aquila, M. E. (2016). Supplementation with nanomolar concentrations of verbascoside during in vitro maturation improves embryo development by protecting the oocyte against oxidative stress: a large animal model study. *Reproductive Toxicology*, *65*, 204–211. https://doi.org/10.1016/j.reprotox.2016.08.004

Mascarenhas, M. N., Flaxman, S. R., Boerma, T., Vanderpoel, S., & Stevens, G. A. (2012). National, Regional, and Global Trends in Infertility Prevalence Since 1990: A Systematic Analysis of 277 Health Surveys. *PLoS Medicine*, *9*(12), 1–12. https://doi.org/10.1371/journal.pmed.1001356

Mattern, F., Herrmann, D., Heinzmann, J., Hadeler, K. G., Bernal-Ulloa, S. M., Haaf, T., & Niemann, H. (2016). DNA methylation and mRNA expression of developmentally

important genes in bovine oocytes collected from donors of different age categories. In *Molecular Reproduction and Development* (Vol. 83, Issue 9). https://doi.org/10.1002/mrd.22692

Mcmanus, M. J., Murphy, M. P., & Franklin, J. L. (2011). The mitochondria-targeted antioxidant mitoq prevents loss of spatial memory retention and early neuropathology in a transgenic mouse model of Alzheimer's disease. *Journal of Neuroscience*, *31*(44), 15703–15715. https://doi.org/10.1523/JNEUROSCI.0552-11.2011

Mulla, A. Al, Fazari, A. B. E., Elkhouly, M., & Moghaddam, N. (2018). Role of Antioxidants in Female Fertility. *Open Journal of Obstetrics and Gynecology*, *08*(02), 85–91. https://doi.org/10.4236/ojog.2018.82011

Munro, D., & Treberg, J. R. (2017). A radical shift in perspective: Mitochondria as regulators of reactive oxygen species. *Journal of Experimental Biology*, *220*(7), 1170–1180. https://doi.org/10.1242/jeb.132142

Nakamura, Y., Tamura, H., Takayama, H., & Kato, H. (2003). Increased endogenous level of melatonin in preovulatory human follicles does not directly influence progesterone production. *Fertility and Sterility*, *80*(4), 1012–1016. https://doi.org/10.1016/S0015-0282(03)01008-2

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*, *109*(2), 501–507.

Nohalez, A., Martinez, C. A., Parrilla, I., Roca, J., Gil, M. A., Rodriguez-Martinez, H., Martinez, E. A., & Cuello, C. (2018). Exogenous ascorbic acid enhances vitrification survival of porcine in vitro-developed blastocysts but fails to improve the in vitro embryo production outcomes. *Theriogenology*, *113*, 113–119. https://doi.org/10.1016/j.theriogenology.2018.02.014

Ombelet, W., & Onofre, J. (2019). New perspective: IVF in Africa: what is it all about ? *Facts Views Vis Obgyn*, *11*(1), 65–76.

Padmanabhan, V., & Veiga-Lopez, A. (2013). Sheep models of polycystic ovary syndrome phenotype. *Molecular and Cellular Endocrinology*, *373*(1–2), 8–20. https://doi.org/10.1016/j.mce.2012.10.005

Paixão, L., Ramos, R. B., Lavarda, A., Morsh, D. M., & Spritzer, P. M. (2017). Animal models of hyperandrogenism and ovarian morphology changes as features of polycystic ovary syndrome: A systematic review. *Reproductive Biology and Endocrinology*, *15*(1), 1–11. https://doi.org/10.1186/s12958-017-0231-z

Palermo, G. D., Neri, Q. V., & Rosenwaks, Z. (2015). To ICSI or not to ICSI. *Seminars in Reproductive Medicine*, *33*(2), 92–102. https://doi.org/10.1055/s-0035-1546825

Pandian, Z., Gibreel, A., & Bhattacharya, S. (2015). In vitro fertilisation for unexplained subfertility. *Cochrane Database of Systematic Reviews*, *2015*(11). https://doi.org/10.1002/14651858.CD003357.pub4

Panth, N., Gavarkovs, A., Tamez, M., & Mattei, J. (2018). The Influence of Diet on Fertility and the Implications for Public Health Nutrition in the United States. *Frontiers in Public Health*, *6*(July), 1–7. https://doi.org/10.3389/fpubh.2018.00211

Penitente Filho, J. M., Oliveira, F. A., & Torres, C. A. A. (2012). *Produção De Embriões Bovinos in Vivo E in Vitro. September*, 20.

Pereira, R. M., Marques, C. C., Baptista, M. de C., Vasques, Ma. I., & Horta, A. E. (2006). Effect of arachidonic acid supplementation and cyclooxygenase / lipoxygenase inhibition on the development of early bovine embryos Influência do ácido araquidónico e da inibição da ciclo-oxigenase ou lipo- oxigenase no dese. *Revista Brasileira de Zootecnia*, *35*(2), 422–427.

Perry, G. (2016). 2015 statistics of embryo collection and transfer in domestic farm animals. *Embryo Transfer Newsl*.

POEGGELER, B., SAARELA, S., REITER, R. J., TAN, D. -X, CHEN, L. -D, MANCHESTER, L. C., & BARLOW-WALDEN, L. R. (1994). Melatonin—A Highly Potent Endogenous Radical Scavenger and Electron Donor: New Aspects of the Oxidation Chemistry of this Indole Accessed in vitro. *Annals of the New York Academy of Sciences*, *738*(1), 419–420. https://doi.org/10.1111/j.1749-6632.1994.tb21831.x

Polge, C., & Rowson, L. E. A. (1952). Fertilizing Capacity of Bull Spermatozoa after Freezing at −79° C. *Nature*, 169.

Putney, D. J., Thatcher, W. W., Drost, M., Wright, J. M., & DeLorenzo, M. A. (1988). Influence of environmental temperature on reproductive performance of bovine embryo donors and recipients in the southwest region of the united states. *Theriogenology*, *30*(5), 905–922. https://doi.org/10.1016/S0093-691X(88)80053-0

R.Pereira, I. Carvalhais, J.Pimenta, M.Baptista, M.Vasques, A.Horta, I.Santos, M.Marques, A.Reis , M Pereira, C. M. (2008). *Biopside and vitrified bovine embryos viability is improved by trans10, cis12 conjugated linoleic acid supplementation during in vitro embryo culture.*

Rahman, M. B., Schellander, K., Luceño, N. L., & Van Soom, A. (2018). Heat stress responses in spermatozoa: Mechanisms and consequences for cattle fertility. *Theriogenology*, *113*, 102–112. https://doi.org/10.1016/j.theriogenology.2018.02.012

Ramalho-Santos, J., Varum, S., Amaral, S., Mota, P. C., Sousa, A. P., & Amaral, A. (2009). Mitochondrial functionality in reproduction: From gonads and gametes to embryos and embryonic stem cells. *Human Reproduction Update*, *15*(5), 553–572. https://doi.org/10.1093/humupd/dmp016

Renzi, A., Di Trani, M., Solano, L., Minutolo, E., & Tambelli, R. (2020). Success of assisted reproductive technology treatment and couple relationship: A pilot study on the role of romantic attachment. *Health Psychology Open*, *7*(1). https://doi.org/10.1177/2055102920933073

Rienzi, L., Gracia, C., Maggiulli, R., Labarbera, A. R., Kaser, D. J., Ubaldi, F. M., Vanderpoel, S., & Racowsky, C. (2017). Oocyte, embryo and blastocyst cryopreservation in art: Systematic review and meta-Analysis comparing slow-freezing versus vitrification to produce evidence for the development of global guidance. *Human Reproduction Update*, *23*(2), 139–155. https://doi.org/10.1093/humupd/dmw038

Rizos, D., Clemente, M., Bermejo-Alvarez, P., De La Fuente, J., Lonergan, P., & Gutiérrez-Adán, A. (2008). Consequences of in vitro culture conditions on embryo development and quality. *Reproduction in Domestic Animals*, *43*(SUPPL.4), 44–50. https://doi.org/10.1111/j.1439-0531.2008.01230.x

Rizos, Dimitrios, Ward, F., Duffy, P., Boland, M. P., & Lonergan, P. (2002). Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: Implications for blastocyst yield and blastocyst quality. *Molecular Reproduction and Development*, *61*(2), 234–248. https://doi.org/10.1002/mrd.1153

Rosa Pereira, C. M. (2017). *Produção e transferência de embriões : uma técnica em expansão* *. 28–29.

Ruder, E. H., Hartman, T. J., & Goldman, M. B. (2009). Impact of oxidative stress on female fertility. *Current Opinion in Obstetrics and Gynecology*, *21*(3), 219–222. https://doi.org/10.1097/GCO.0b013e32832924ba

Santos, J. P. C. dos. (2020). *Impacts of novel mitochondrial-directed antioxidant on bovine sperm function and in vitro embryo development*. Escola Universitária Vasco da Gama.

Scherz-Shouval, R., & Elazar, Z. (2011). Regulation of autophagy by ROS: Physiology and pathology. *Trends in Biochemical Sciences*, *36*(1), 30–38. https://doi.org/10.1016/j.tibs.2010.07.007

Schofield, J. H., & Schafer, Z. T. (2021). Mitochondrial Reactive Oxygen Species and Mitophagy: A Complex and Nuanced Relationship. *Antioxidants and Redox Signaling*, *34*(7), 517–530. https://doi.org/10.1089/ars.2020.8058

Sena, L. A., & Chandel, N. S. (2012). Physiological roles of mitochondrial reactive oxygen species. *Molecular Cell*, *48*(2), 158–167. https://doi.org/10.1016/j.molcel.2012.09.025

Sharlip, I. D., Jarow, J. P., Belker, A. M., Lipshultz, L. I., Sigman, M., Thomas, A. J., Schlegel, P. N., Howards, S. S., Nehra, A., Damewood, M. D., Overstreet, J. W., & Sadovsky, R. (2002). Best practice policies for male infertility. *Fertility and Sterility*, *77*(5), 873–882. https://doi.org/10.1016/S0015-0282(02)03105-9

Sharma, R. K., & Agarwal, A. (1996). Role of reactive oxygen species in male infertility. *Urology*, *48*(6), 835–850. https://doi.org/10.1016/S0090-4295(96)00313-5

Shear, M. A., Vaughan, D. A., Modest, A. M., Seidler, E. A., Leung, A. Q., Hacker, M. R., Sakkas, D., & Penzias, A. S. (2020). Blasts from the past: is morphology useful in PGT-A tested and untested frozen embryo transfers? *Reproductive BioMedicine Online*, *41*(6), 981–989. https://doi.org/10.1016/j.rbmo.2020.07.014

Shi, X., & Qiu, H. (2020). Post-translational S-nitrosylation of proteins in regulating cardiac oxidative stress. *Antioxidants*, *9*(11), 1–23. https://doi.org/10.3390/antiox9111051

Silber, S. J., Kato, K., Aoyama, N., Yabuuchi, A., Skaletsky, H., Fan, Y., Shinohara, K., Yatabe, N., & Kobayashi, T. (2017). Intrinsic fertility of human oocytes. *Fertility and Sterility*, *107*(5), 1232–1237. https://doi.org/10.1016/j.fertnstert.2017.03.014

Silva, M. do C. (2015). *Criopreservação de oócitos : efeito de diferentes protocolos na viabilidade pós-vitrificação*. Universidade de Lisboa Faculdade de Ciências.

Somigliana, E., Busnelli, A., Vigano, P., Vegetti, W., Goisis, A., & Vercellini, P. (2020). Is early initiation of infertility treatment justified in women over the age of 35 years? *Reproductive BioMedicine Online*, *40*(3), 393–398. https://doi.org/10.1016/j.rbmo.2019.12.006

Son, S., & Lewis, B. A. (2002). Free radical scavenging and antioxidative activity of caffeic

acid amide and ester analogues: Structure-activity relationship. *Journal of Agricultural and Food Chemistry*, *50*(3), 468–472. https://doi.org/10.1021/jf010830b

Soncin, F., Natale, D., & Parast, M. M. (2015). Signaling pathways in mouse and human trophoblast differentiation: A comparative review. *Cellular and Molecular Life Sciences*, *72*(7), 1291–1302. https://doi.org/10.1007/s00018-014-1794-x

Soto-Heras, S., & Paramio, M. T. (2020). Impact of oxidative stress on oocyte competence for in vitro embryo production programs. *Research in Veterinary Science*, *132*(May), 342–350. https://doi.org/10.1016/j.rvsc.2020.07.013

Sousa, J. S., D'Imprima, E., & Vonck, J. (2018). Mitochondrial respiratory chain complexes. In *Subcellular Biochemistry* (Vol. 87). https://doi.org/10.1007/978-981-10-7757-9_7

Springer, C., Wolf, E., & Simmet, K. (2021). A new toolbox in experimental embryology—alternative model organisms for studying preimplantation development. *Journal of Developmental Biology*, *9*(2). https://doi.org/10.3390/jdb9020015

Steptoe, P. C., Edwards, R. G., & Purdy, J. M. (1971). Human blastocysts grown in culture. *Nature*, *229*(5280), 132–133. https://doi.org/10.1038/229132a0

Sun, H., Gong, T. T., Jiang, Y. T., Zhang, S., Zhao, Y. H., & Wu, Q. J. (2019). Global, Regional, and National Prevalence and Disability-Adjusted Life-Years for Infertility in 195 Countries and Territories, 1990-2017: Results from a Global Burden of Disease Study, 2017. *Aging*, *11*(23), 10952–10991. https://doi.org/10.18632/aging.102497

Sun, W. J., Pang, Y. W., Liu, Y., Hao, H. S., Zhao, X. M., Qin, T., Zhu, H. Bin, & Du, W. H. (2015). Exogenous glutathione supplementation in culture medium improves the bovine embryo development after in vitro fertilization. *Theriogenology*, *84*(5), 716–723. https://doi.org/10.1016/j.theriogenology.2015.05.001

Sun, Y., Zhou, K., He, M., Gao, Y., Zhang, D., Bai, Y., Lai, Y., Liu, M., Han, X., Xu, S., Tian, W., & Xu, J. (2020). The Effects of Different Fluorescent Indicators in Observing the Changes of the Mitochondrial Membrane Potential during Oxidative Stress-Induced Mitochondrial Injury of Cardiac H9c2 Cells. *Journal of Fluorescence*, *30*(6), 1421–1430. https://doi.org/10.1007/s10895-020-02623-x

Szeto, H. H. (2008). Mitochondria-targeted cytoprotective peptides for ischemia-reperfusion injury. *Antioxidants and Redox Signaling*, *10*(3), 601–619. https://doi.org/10.1089/ars.2007.1892

Szkodziak, P., Wozniak, S., Czuczwar, P., Wozniakowska, E., Milart, P., Mroczkowski, A., & Paszkowski, T. (2016). Infertility in the light of new scientific reports – Focus on male factor. *Annals of Agricultural and Environmental Medicine*, *23*(2), 227–230. https://doi.org/10.5604/12321966.1203881

Tay, J. I., Rutherford, A. J., Killick, S. R., Maguiness, S. D., Partridge, R. J., & Leese, H. J. (1997). Human tubal fluid: Production, nutrient composition and response to adrenergic agents. *Human Reproduction*, *12*(11), 2451–2456. https://doi.org/10.1093/humrep/12.11.2451

Teixeira, C., Marques, C. C., Baptista, M. C., Pimenta, J., Teixeira, J., Cagide, F., Borges, F., Montezinho, L., Oliveira, P., & Pereira, R. M. L. N. (2020). *Beneficial effect of mitochondriotropic antioxidants on oocyte maturation and embryo production*.

Teixeira, Jose, Cagide, F., Benfeito, S., Soares, P., Garrido, J., Baldeiras, I., Ribeiro, J. A., Pereira, C. M., Silva, A. F., Andrade, P. B., Oliveira, P. J., & Borges, F. (2017). Development of a mitochondriotropic antioxidant based on caffeic acid: Proof of concept on cellular and mitochondrial oxidative stress models. *Journal of Medicinal Chemistry*, *60*(16), 7084–7098. https://doi.org/10.1021/acs.jmedchem.7b00741

Teixeira, José, Oliveira, C., Amorim, R., Cagide, F., Garrido, J., Ribeiro, J. A., Pereira, C. M., Silva, A. F., Andrade, P. B., Oliveira, P. J., & Borges, F. (2017). Development of hydroxybenzoic-based platforms as a solution to deliver dietary antioxidants to mitochondria. *Scientific Reports*, *7*(1), 1–18. https://doi.org/10.1038/s41598-017-07272-y

Teixeira, José, Oliveira, C., Cagide, F., Amorim, R., Garrido, J., Borges, F., & Oliveira, P. J. (2018). Discovery of a new mitochondria permeability transition pore (mPTP) inhibitor based on gallic acid. *Journal of Enzyme Inhibition and Medicinal Chemistry*, *33*(1), 567–576. https://doi.org/10.1080/14756366.2018.1442831

Teixeira, José, Soares, P., Benfeito, S., Gaspar, A., Garrido, J., Murphy, M. P., & Borges, F. (2012). Rational discovery and development of a mitochondria-targeted antioxidant based on cinnamic acid scaffold. *Free Radical Research*, *46*(5), 600–611. https://doi.org/10.3109/10715762.2012.662593

Ter Keurst, A., Boivin, J., & Gameiro, S. (2016). Women's intentions to use fertility preservation to prevent age-related fertility decline. *Reproductive BioMedicine Online*, *32*(1), 121–131. https://doi.org/10.1016/j.rbmo.2015.10.007

Thompson, J. G., McNaughton, C., Gasparrini, B., McGowan, L. T., & Tervit, H. R. (2000). Effect of inhibitors and uncouplers of oxidative phosphorylation during compaction and blastulation of bovine embryos cultured in vitro. *Journal of Reproduction and Fertility*, *118*(1), 47–55. https://doi.org/10.1530/jrf.0.1180047

Torres, V., Hamdi, M., Maillo, V., Urrego, R., Echeverri, J. J., López-Herrera, A., Gutiérrez-Adán, A., Rizos, D., & Sánchez-Calabuig, M. J. (2019). Ascorbic acid–cyclodextrin complex alters the expression of genes associated with lipid metabolism in bovine in vitro produced embryos. *Reproduction in Domestic Animals*, *54*(1), 55–62. https://doi.org/10.1111/rda.13311

Trevizan, J. T., Carreira, J. T., Carvalho, I. R., Kipper, B. H., Nagata, W. B., Perri, S. H. V., Franco Oliveira, M. E., Pierucci, J. C., & Koivisto, M. B. de. (2018). Does lipid peroxidation and oxidative DNA damage differ in cryopreserved semen samples from young, adult and aged Nellore bulls? *Animal Reproduction Science*, *195*(November 2017), 8–15. https://doi.org/10.1016/j.anireprosci.2018.04.071

Trounson, A., & Gardner, D. K. (1993). *Handbook of In Vitro Fertilization* (D. K. G. Alan Trounson (ed.); CRC Press).

Truong, T. T., Soh, Y. M., & Gardner, D. K. (2016). Antioxidants improve mouse preimplantation embryo development and viability. *Human Reproduction*, *31*(7), 1445–1454. https://doi.org/10.1093/humrep/dew098

Veal, E. A., Day, A. M., & Morgan, B. A. (2007). Hydrogen Peroxide Sensing and Signaling. *Molecular Cell*, *26*(1), 1–14. https://doi.org/10.1016/j.molcel.2007.03.016

Veloso, C. D. (2019). *Mitochondriotropic Antioxidants Based on Dietary Phenolic Acids as Modulators of Oxidative Stress on Skin Fibroblasts*. Universidade de Coimbra.

Wagner, H., Cheng, J. W., & Ko, E. Y. (2018). Role of reactive oxygen species in male infertility: An updated review of literature. *Arab Journal of Urology*, *16*(1), 35–43. https://doi.org/10.1016/j.aju.2017.11.001

Wong, K. M., van Wely, M., Verhoeve, H. R., Kaaijk, E. M., Mol, F., van der Veen, F., Repping, S., & Mastenbroek, S. (2021). Transfer of fresh or frozen embryos: a randomised controlled trial. *Human Reproduction (Oxford, England)*, *36*(4), 998–1006. https://doi.org/10.1093/humrep/deaa305

Yang, M., Tao, J., Chai, M., Wu, H., Wang, J., Li, G., He, C., Xie, L., Ji, P., Dai, Y., Yang, L., & Liu, G. (2017). Melatonin improves the quality of inferior bovine oocytes and promoted their subsequent IVF embryo development: Mechanisms and results. *Molecules*, *22*(12). https://doi.org/10.3390/molecules22122059

Zegers-Hochschild, F., Adamson, G. D., de Mouzon, J., Ishihara, O., Mansour, R., Nygren, K., Sullivan, E., & Vanderpoel, S. (2009). International Committee for Monitoring Assisted Reproductive Technology (ICMART) and the World Health Organization (WHO) revised glossary of ART terminology, 2009*. *Fertility and Sterility*, *92*(5), 1520–1524. https://doi.org/10.1016/j.fertnstert.2009.09.009

Zhang, H. M., & Zhang, Y. (2014). Melatonin: A well-documented antioxidant with conditional pro-oxidant actions. *Journal of Pineal Research*, *57*(2), 131–146. https://doi.org/10.1111/jpi.12162

Zhang, L., & Demain, A. L. (2005). Natural products and drug discovery. *Natural Products: Drug Discovery and Therapeutic Medicine*, 3–29. https://doi.org/10.1007/978-1-59259-976-9_1

Zolini, A. M., Carrascal-Triana, E., Ruiz de King, A., Hansen, P. J., Alves Torres, C. A., & Block, J. (2019). Effect of addition of L-carnitine to media for oocyte maturation and embryo culture on development and cryotolerance of bovine embryos produced in vitro. *Theriogenology*, *133*, 135–143. https://doi.org/10.1016/j.theriogenology.2019.05.005