ESCOLA UNIVERSITÁRIA VASCO DA GAMA

MESTRADO INTEGRADO EM MEDICINA VETERINÁRIA

Impacts of a novel mitochondrial-directed antioxidant on bovine sperm function and *in vitro* embryo development

João Pedro Campos dos Santos

Coimbra, julho 2020

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List of abbreviations (alphabetic order)

ALH: amplitude of lateral head displacement

ART: assisted reproductive techniques

BCF: beat cross frequency

BL: blastocysts

BSA: bovine serum albumin

CAP: capacitation medium

CASA: Computer-Assisted Sperm Analysis

COC: cumulus oocyte complexes

EBL: expanded blastocysts

FCS: fetal calf serum

FERT: fertilization medium

HBA: hydroxybenzoic acid

HBL: hatched blastocysts

IETS: International Embryo Technology Society

LIN: linearity

M: morulae

PBS: phosphate buffered saline

ROS: reactive oxygen species

SOF: synthetic oviductal fluid

STR: straightness

TCM: tissue culture medium 199

VAP: average path velocity

VCL: curvilinear velocity

VSL: straight-line velocity

YBL: young blastocyst

Impacts of a novel mitochondrial-directed antioxidant on bovine sperm function and *in vitro* embryo development

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1. Resumo

Os espermatozóides são particularmente vulneráveis às espécies reativas de oxigénio nas diferentes fases do processo reprodutivo (maturação, capacitação, hiperativação, reação acrossomal). Por esta razão, este trabalho teve como objetivo o estudo do efeito de um inovador antioxidante dirigido às mitocôndrias (AntiOxBEN2) na função espermática e no desenvolvimento embrionário *in vitro* bovino. Este antioxidante foi adicionado ao meio de capacitação espermática (CAP), durante o processo de *swim-up,* e ao meio de fertilização (FERT), durante a co-incubação dos oócitos maturados com o sémen capacitado, nas concentrações de 0 (controlo), 1 e 10 µM. Foram assim constituídos 7 grupos: (1): CAPcontrolo x FERTcontrolo; (2): CAPcontrolo x FERT1; (3): CAPcontrolo x FERT10; (4): CAP1 x FERTcontrolo; (5): CAP1 x FERT1; (6): CAP10 x FERTcontrolo; (7): CAP10 x FERT 10. Após o *swim-up* foi avaliada a motilidade espermática (CASA e análise visual), a vitalidade (eosina-negrosina), o potencial de membrana mitocondrial (JC1) e o metabolismo basal (Seahorse Xfe96) dos espermatozoides. Foi igualmente avaliado o desenvolvimento e a qualidade dos embriões produzidos. O grupo CAP1 x FERT1 apresentou a maior taxa de clivagem, comparativamente ao grupo controlo ($p<0.04$). Foi identificado um efeito positivo do AntiOxBEN2 no potencial de membrana mitocondrial ($p\leq0.003$) e, consequentemente, na qualidade espermática, enquanto que a concentração mais elevada prejudicou a motilidade progressiva e o número de embriões produzidos. Os resultados demonstraram um efeito benéfico do AntiOxBEN2 (na concentração de 1 µM) nos processos de capacitação espermática e na fertilização, melhorando o desenvolvimento embrionário.

Palavras-chave (ordem alfabética)

Antioxidante dirigido à mitocôndria, bovino, capacitação de sémen, desenvolvimento embrionário *in vitro*, espécies reativas de oxigénio, fertilização, stress oxidativo.

2. Abstract

Sperm cells are particularly vulnerable to reactive oxygen species at different stages of the reproductive process (maturation, capacitation, hyperactivation and acrosomal reaction). For this reason, our objective was to study the effect of a novel mitochondrial-directed antioxidant, AntiOxBEN2, on bovine sperm function and *in vitro* embryo development. This antioxidant was added to the semen capacitation medium (CAP), during the swim-up, and to the fertilization medium (FERT) during the co-incubation of matured oocytes and capacitated spermatozoa, in concentrations of 0 (control), 1 and 10 µM. Thus, 7 groups were constituted: (1): CAPcontrol x FERTcontrol; (2): CAPcontrol x FERT1; (3): CAPcontrol x FERT10; (4): CAP1 x FERTcontrol; (5): CAP1 x FERT1; (6): CAP10 x FERTcontrol; (7): CAP10 x FERT 10. After the swim-up, sperm motility (CASA and visual analysis), vitality (eosin-nigrosin), mitochondrial membrane potential (JC1) and basal metabolism (Seahorse Xfe96) were evaluated. Embryonic development and quality of produced embryos were assessed. Higher cleavage rate was obtained in CAP1 x FERT1 compared to control ($p<0.04$). A positive effect of AntiOxBEN2 on the mitochondrial membrane potential ($p\leq0.003$) and, consequently, on the sperm quality was identified, although the highest dose impaired progressive motility and the number of produced embryos. The results demonstrate a beneficial effect of AntiOxBEN2 (1 µM) on sperm capacitation and fertilization processes, thus improving embryonic development.

Key words (alphabetic order)

Bovine, fertilization, *in vitro* embryo development, mitochondrial-directed antioxidant, oxidative stress, reactive oxygen species, sperm capacitation.

3. Introduction

Physiologically, in eukaryotic cells of any species, reactive oxygen species (ROS) are in balance with the presence of antioxidants, and this balance is essential for maintaining functionality. When there is an imbalance in this system, a phenomenon called oxidative stress occurs (Valera-Alberni & Canto, 2018). In germline most ROS are formed in mitochondria, where oxidative phosphorylation and the consequent reduction of molecular oxygen take place for the production of energy, fundamental to life (Aitken et al., 2016). This oxygen reduction can be incomplete, thus forming ROS, such as the superoxide anion (O2•-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH•). The main antioxidant enzymes present in cells and responsible for the conversion of ROS into harmless substances are superoxide dismutase, catalase and the glutathione peroxidase/reductase system (Valera-Alberni & Canto, 2018). When there is an elevation in ROS, damage may occur in several essential biomolecules, such as lipids, polysaccharides, proteins and DNA, which can consequently lead to cell death by apoptosis/necrosis (Khan, 2013; Teixeira et al., 2017). Depending on where the imbalance in the production/elimination of ROS occurs, oxidative stress can have several repercussions. For instance, in the reproductive system, it can affect fertility and consequently reproductive ability (Martin-Hidalgo et al., 2019).

Sperm functioning requires the production of a large amount of ATP and thus of mitochondria, which inevitably may lead to an increased production of ROS (Martin-Hidalgo et al., 2019). Sperm cells are particularly vulnerable to ROS at different stages of the reproductive process (maturation, capacitation, hyperactivation and acrosomal reaction) (Otasevic et al., 2019). Changes in spermatozoa's plasma membranes, as well as a decrease in their motility and vitality have been reported, when in the presence of high levels of ROS (Martin-Hidalgo et al., 2019; Panner Selvam et al., 2020). If low levels of disequilibrium between ROS and cellular defenses exist, sperm cells can maintain their fertilization ability. However, when a hard damage occurs, it can be transmitted to the next generation (Aitken & Baker, 2006). Special attention should be given to the assisted reproductive techniques (ART), in order to reduce the impact of ROS on the number and quality of produced embryos, given the harmful effect they have on both oocytes and spermatozoa (De Assis et al., 2015). Several factors can increase the oxidative stress during the *in vitro* process, such as: light, oxygen concentration and the handling of oocytes, sperm and embryos (Bermejo-Álvarez et al., 2010; Khazaei & Aghaz, 2017; Leite et al., 2017). Therefore, an efficient solution to control oxidative stress should be attempted to accomplish ART successfully.

AntiOxBEN2 is a derivative of hydroxybenzoic acid (HBA), which is a subclass of phenolic acids, the main metabolites of polyphenols. HBA is present in relatively high amounts in plants and fruits, and have several biological properties (antioxidant, anti-inflammatory, antimicrobial, anticancer and neuroprotective) (Heleno et al., 2015; Pezzini et al., 2017; Szwajgier et al., 2017). The antioxidant activity of HBAs has been associated with their chelating and scavenging properties of ROS, acting in the prevention of lipid peroxidation and in the inhibition of pro-oxidant enzymes involved in the production of ROS. The use of HBAs in human and veterinary therapy, alone or as adjuvants, is

restricted due to limitations related to their bioavailability, pharmacokinetics, and selectivity, which are linked to their physicochemical properties (e.g. low lipophilicity) and fast metabolism. Several studies have been carried out to increase the lipophilicity and stability of HBAs, as well, as to improve their availability for intracellular targets.

The recognition of the essential role of mitochondria in the maintenance of cell life and the mitochondrial dysfunction observed in several pathological states (Smith et al., 2012), encouraged the study of new drugs focused on mitochondrial pharmacology. Despite this, the discovery and use of potential mitochondrial drugs is a challenge because they require a specific target affinity (drug delivery to mitochondria) and the guarantee that they do not present mitochondrial toxicity. The antioxidant molecule, AntiOxBEN2, resulted from this research presenting high lipophilicity, stability, and selectivity for the mitochondria, being therefore designated as mitochondrial directed (Oliveira et al., 2018; Teixeira et al., 2017). This compound has already been reported to have positive effects in other experimental tests performed using other cell models (Oliveira et al., 2018; Teixeira et al., 2017) and preliminary results indicate that this molecule improves the rates of oocyte maturation and embryo cleavage, when supplemented to the medium of maturation (Teixeira et al., 2020).

A growing interest in controlling oxidative stress and its implication in numerous conditions, such as fertility, justified the need to evaluate new agents capable of modulating spermatozoa functioning. This project is quite innovative since, to date, there are no published data in this area In this study we investigated, for the first time, the effect of a novel mitochondrial-directed antioxidant molecule, the AntiOxBEN2 (Oliveira et al., 2018; Teixeira et al., 2017) in the prevention of oxidative stress of bovine spermatozoa during both the capacitation and fertilization processes. Spermatozoa motility, vitality, oxygen consumption and the mitochondrial membrane potential as well as their fertilization and embryonic developmental ability were assessed.

4. Material and Methods

4.1 Experimental design

Our goal was to evaluate the effect of a novel mitochondrial-directed antioxidant molecule, AntiOxBEN2 (Teixeira et al., 2017), on both bovine sperm capacitation and fertilization processes and subsequent embryonic development. This molecule was added to the capacitation medium (CAP), at the concentration of 0 (control), 1 and 10 µM, where the thawed bovine spermatozoa were submitted to the swim-up process for 1 hour and then evaluated prior to the fertilization process. The same concentrations of AntiOxBEN2 were also supplemented to the fertilization medium (FERT), during the co-incubation of *in vitro* matured oocytes and capacitated spermatozoa, giving rise to 7 groups as follows: (1): CAPcontrol x FERTcontrol - FERT and CAP without supplementation; (2): CAPcontrol x FERT1 - CAP without supplementation and FERT with 1 µM of AntiOxBEN2; (3): CAPcontrol x FERT10 - CAP without supplementation and FERT with 10 µM AntiOxBEN2; (4): CAP1 x FERTcontrol – CAP with 1 µM of AntiOxBEN2 and FERT without supplementation; (5): CAP1 x FERT1 - CAP and FERT with 1 µM of AntiOxBEN2; (6): CAP10 x FERTcontrol - CAP with 10 µM of AntiOxBEN2 and FERT without supplementation; and finally (7): CAP10 x FERT 10 – CAP and FERT with 10 µM of AntiOxBEN2.

Bovine oocytes (n=1864) collected in 6 sessions were previously matured for 22 hours and randomly distributed into the 7 groups prior to insemination with the capacitated spermatozoa. Sperm from two bulls were used and after the swim-up process its motility [visual and computer-assisted sperm analysis (CASA)], vitality (eosin-nigrosin), mitochondrial membrane potential (JC1) and function (basal metabolism, Seahorse Xfe96) were evaluated. After transferring the presumptive zygotes, the embryonic development was evaluated by the cleavage, day 7 and hatched rates and quality of produced embryos.

4.2 Oocyte collection and *in vitro* maturation

Bovine ovaries were harvested at the local slaughterhouse, immediately after slaughter, and were placed in Dulbecco's Phosphate Buffered Saline (PBS), at 38 ºC, to be transported to the laboratory. PBS was previously supplemented with 0.15 % bovine serum albumin (BSA, w/v) and 0.05 $mgmL^{-1}$ kanamycin. Upon reaching the laboratory, the ovaries were washed, and the cumulus oocyte complexes (COC) of the follicles with a diameter between 2-6 mm were aspirated. Then oocytes with uniformly granular cytoplasm and surrounded by at least 3 layers of compact cumulus cells were selected for maturation (Lapa et al., 2011).

The COCs were matured in tissue culture medium 199 (TCM199) with 10 % serum, 10 ng mL^{-1} epidermal growth factor, 0.2 mM sodium pyruvate and antibiotics, in an incubator at 38.8 ºC with an atmosphere saturated with humidity and 5 % CO2, for 22 hours (Fonseca et al., 2020).

4.3 Semen capacitation and oocyte *in vitro* fertilization

In order for bovine spermatozoa to have the ability to penetrate and fertilize the oocyte, they have to undergo a maturation process called sperm capacitation (Moore & Hasler, 2017). For this, the Swim-up method was used. Frozen semen straws from two bulls of proven fertility were thawed at 37 ºC for 30 seconds. Then spermatozoa were submitted to swim-up process in capacitation medium [modified Tyrode's medium without calcium supplemented with 2.4 mM of caffeine (Pereira et al., 2006)], supplemented or not with the antioxidant molecule according to the experimental design. Briefly, thawed semen was carefully deposited, in equal doses, on the bottom of the previously prepared capacitation medium-containing tubes and placed in the incubator for 1 hour, in an atmosphere of 5 % CO2, at 38.8 ºC and with maximum humidity. During this period, the swim-up occurred, a process by which the most active spermatozoa migrate to the surface of the liquid, concentrating there. Then, the upper layer was centrifuged at 225 x*g* for 10 minutes and the supernatant rejected. The remaining pellet of spermatozoa was evaluated prior to be used to fertilize the oocytes in fertilization medium.

Post swim-up spermatozoa motility (visual and CASA analysis), vitality (eosin-nigrosin dye), mitochondrial polarization (JC1, fluorescent dye) and the basal metabolism of mitochondria (Seahorse Xfe96) were evaluated (adapted from Ferreira et al., 2017, Fonseca et al., 2020 and Deus et al., 2015). The kinematic parameters evaluated with CASA were: total and progressive motile (%), average path velocity (VAP, μms^{-1}), straight-line velocity (VSL, μms^{-1}), curvilinear velocity (VCL, μms^{-1}) amplitude of lateral head displacement (ALH, µm), beat cross frequency (BCF, Hz), straightness, linearity, rapid, medium, slow and static motile sperm. For evaluation of basal metabolism of mitochondria, spermatozoa (CAPcontrol, CAP1 and CAP10) were centrifuged and placed in 96-well plate in SOF medium at a density of 1×10^6 cells/100µL/well (8 wells each group). After incubation time, oxygen consumption was measured at 38.5 ºC using a Seahorse XFe96 Extracellular Flux Analyzer (Seahorse Bioscience, Germany) (Deus et al., 2015).

Spermatozoa (2×10^6 $spzmL^{-1}$) were added to droplets of fertilization medium (40 µL), containing 10 oocytes each. The *in vitro* fertilization (IVF) medium consisted of modified Tyrode's medium supplemented with 5.4 USP mL^{-1} of heparin, 10 mM of penicillamine, 20 mM of hypotaurine and 0.25 mM of epinephrine (Fonseca et al., 2020) and was also supplemented or not with AntiOxBEN2 according to the experimental design. The spermatozoa and oocytes were co-incubated for 20 hours.

4.4 Embryo culture and evaluation

Twenty hours after insemination, denuded presumptive zygotes were placed in 25 µL droplets of synthetic oviductal fluid (SOF) supplemented with MEM non-essential Amino Acid Solution, BME Amino acids solution, glutathione and BSA, and cultured at 38.8 ºC in a humidified atmosphere with 5 % O2, 5 % CO2 and 90 % N2.

After assessing cleavage at 48 hours post insemination, embryo development proceeded in SOF BSA plus 10 % fetal calf serum (FCS) (IVF = day 0) for ten days in the incubator at the same conditions. Embryo development was assessed. Cleavage (2–4 cell embryos) rates were calculated as

a proportion of inseminated oocytes, D7/8 embryo (morula and blastocysts, with most embryos being at the blastocyst stage) developmental rates were calculated as a proportion of cleaved embryos, while the rates of hatched embryos were calculated as a proportion of embryos of day 7/8 that effectively hatched from the zona pellucida until D12 (Pereira et al., 2009).

Moreover, D7 embryos were classified as good, fair and bad according to International Embryo Technology Society guidelines (Stringfellow & Seidel, 1998).

The rate of embryonic development was also assessed on D7 and 9, identifying morulae (M), young blastocysts (YBL), blastocysts (BL), expanded blastocysts (EBL) and hatched blastocysts (HBL) (Pereira et al., 2009).

4.5 Statistical analysis

The procedure MIXED of Statistical Analysis Systems Institute (SAS Inst., Inc., Cary, NC, USA) was used to analyze data from sperm parameters. The mixed linear model included the treatment and bull as fixed effects. In addition, the means for each treatment were calculated and compared. Embryo production data were analyzed with the procedure GLIMMIX using the binary distribution and the logit as link function. The generalized linear mixed model included treatment and session as fixed and random effects, respectively. Values were considered statistically different when $p \leq 0.05$.

5. Results

5.1 Evaluation of semen kinematic parameters

After thawing bull semen was immediately evaluated and then submitted to the swim-up process in the capacitation medium. The results of the semen kinematic parameters were represented in Table 1. The presence of AntiOXBEN2 during capacitation influenced the progressive motility of spermatozoa ($p=0.03$) evaluated by using CASA. This parameter was decreased by the highest dose (10 µM) when compared to control ($p=0.009$, Table 1). However, when comparing to 1 µM supplementation, the spermatozoa motility tended ($p=0.0595$) also to be lower in the highest dose. Equally, an effect ($p=0.004$) of the treatment on visual spermatozoa individual motility was identified with the highest dose impairing this motility compared to 1 µM ($p=0.04$) and control ($p=0.001$) groups (Table 1).

A bull effect was observed in the number of sperm classified with medium velocity ($p=0.02$). All other measured parameters (Table 1) showed no differences, neither for the bull nor for the treatment with the molecule under study.

Table 1. Evaluation of the effect of AntiOxBEN2 on the kinematic parameters of semen using Computer Assisted Sperm Analysis (CASA) software and visual observation.

Kinematic parameters	Groups		
	CAPcontrol	**CAP1**	**CAP10**
Total Motility (%)	79.6 ± 7.03	75.8 ± 7.03	69.8 ± 7.03
Progressive Motility (%)	42.8 ± 5.85 **a**	37.9 ± 5.85 **ab**	28.5 ± 5.85 **b**
Visual Motility (%)	56.5 ± 5.16 **a**	50.7 ± 5.16 **a**	44.0 ± 5.16 **b**
VCL (µms^{-1})	114.6 ± 14.4	99.5 ± 14.4	97.5 ± 14.4
VAP (µms^{-1})	59.1 ± 7.02	51.6 ± 7.02	50.5 ± 7.02
Static motile sp (n)	36.9 ± 11.52	40.9 ± 11.52	55.3 ± 11.52
Slow motile sp (n)	4.9 ± 2.47	8.3 ± 2.47	11.8 ± 2.47
Medium motile sp (n)	20.9 ± 6.05	26.4 ± 6.05	25.1 ± 6.05
Rapid motile sp (n)	145.1 ± 35.16	136.4 ± 35.16	92.1 ± 35.16
VSL (µms^{-1})	45.2 ± 6.21	37.4 ± 6.21	35.6 ± 6.21
ALH (µm)	2.8 ± 0.30	2.5 ± 0.30	2.6 ± 0.30
Linearity	39.1 ± 1.95	37.0 ± 1.95	37.3 ± 1.95
Straightness	75.4 ± 2.72	71.6 ± 2.72	70.8 ± 2.72
Wobble VAP/VCL	51.8 ± 1.23	51.5 ± 1.23	52.5 ± 1.23
BCF (Hz)	13.5 ± 1.18	13.3 ± 1.18	11.9 ± 1.18

Different letters indicate significant differences ($p \leq 0.05$). VCL: curvilinear velocity; VAP: average path velocity; sp: sperm; VSL: straight-line velocity; ALH: amplitude of lateral head displacement; BCF: beat cross frequency. CAPcontrol: capacitation medium without supplementation; CAP1: capacitation medium supplemented with 1 µM of AntiOxBEN2; CAP10: capacitation medium supplemented with 10 µM of AntiOxBEN2.

5.2 Evaluation of semen vitality, abnormalities, oxygen consumption and mitochondrial membrane potential

The supplementation of spermatozoa during capacitation with different concentrations of AntiOxBEN2 showed no significant differences (p>0.05) in sperm vitality, normality, and head, intermediate piece, or tail defect rates (Table 2). The bull had no significant effect in any of these rates (p>0.05).

On the contrary, a significant effect (p=0.003, Table 2) of the AntiOxBEN2 on mitochondrial membrane potential was identified. Both doses, 1 µM (p=0.003), and 10 µM (p=0.002) of AntiOxBEN2 improved the number of spermatozoa emitting a bright red-orange fluorescence when compared to control group. No differences were identified between bulls (p>0.05). Moreover, oxygen consumption of spermatozoa seems to decrease in a dose dependent manner (Figure 1).

Table 2. Sperm vitality, head, intermediate piece and tail anomalies, and mitochondrial membrane potential after the capacitation with AntiOxBEN2.

Parameters	Groups		
	CAPcontrol	**CAP1**	**CAP10**
Vitality (%)	47.3 ± 6.96	44.9 ± 6.96	45.8 ± 6.96
Normal spz (%)	79.8 ± 1.65	81.2 ± 1.65	80.5 ± 1.65
Head defect (%)	7.3 ± 0.84	7.4 ± 0.84	9.1 ± 0.84
Int.piece defect (%)	6.8 ± 1.17	5.8 ± 1.17	5.1 ± 1.17
Tail defect (%)	6.7 ± 0.88	6.2 ± 0.88	5.8 ± 0.88
Red-orange fluorescence (%)	67.2 ± 5.79 **b**	82.7 ± 5.79 **a**	83.5 ± 5.79 **a**
Green fluorescence (%)	32.8 ± 5.79 **a**	17.3 ± 5.79 **b**	16.5 ± 5.79 **b**

Different letters indicate significant differences (p≤0.05); CAPcontrol: capacitation medium without supplementation; CAP1: capacitation medium supplemented with 1 µM of AntiOxBEN2; CAP10: capacitation medium supplemented with 10 µM of AntiOxBEN2;

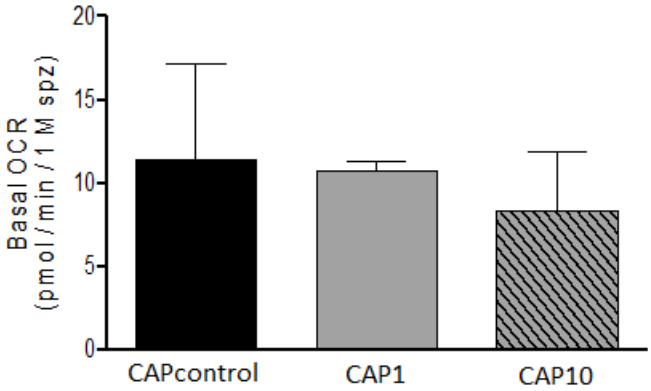

Figure 1. Basal oxygen consumption rate (OCR) of spermatozoa analyzed by Seahorse XF96 Extracellular Flux Analyzer. CAPcontrol: capacitation medium without supplementation; CAP1: capacitation medium supplemented with 1 µM of AntiOxBEN2; CAP10: capacitation medium supplemented with 10 µM of AntiOxBEN2.

5.3 Embryonic development

The supplementation of both the capacitation and fertilization media with AntiOxBEN2 influenced the cleavage rate (p=0.02, Table 3), but had no significant effect in the embryo production rate at Day 7 (p>0.05), nor in the hatched embryo rate (p>0.05). Nevertheless, the supplementation of both media with 1µM of AntiOxBEN2 more than doubled the number of D7 produced embryos compared to the 10 µM dose.

The CAP1 x FERT1 group showed a higher cleavage rate when compared to the control CAPcontrol x FERTcontrol (p=0.01), CAP10 x FERTcontrol (p=0.046), CAP1 x FERTcontrol (p=0.01), and CAPcontrol x FERT10 (p=0.001) groups. Moreover, CAPcontrol x FERT1 and CAP10 x FERT10 also had higher (p≤0.04) cleavages rates when compared to CAPcontrol x FERT10 (Table 3).

Table 3. Effect of oocytes and semen treatment with different concentrations of AntiOxBEN2 on cleavage and embryo production rates.

Groups	Inseminated oocytes (n)	Cleavage (%)	D7embryos (n)	D7 embryos (%)
CAPcontrol x FERTcontrol	306	61.8 ± 2.84 **bc**	28	13.6 ± 2.47
CAPcontrol x FERT1	266	69.4 ± 2.86 **ab**	31	16.2 ± 2.73
CAPcontrol x FERT10	243	57.2 ± 3.22 **c**	15	10.3 ± 2.56
CAP1 x FERTcontrol	248	61.4 ± 3.14 **bc**	27	16.3 ± 2.97
CAP1 x FERT1	264	72.7 ± 2.77 **a**	38	18.7 ± 2.83
CAP10 x FERTcontrol	293	64.4 ± 2.85 **bc**	22	11.0 ± 2.26
CAP10 x FERT10	244	66.9 ± 3.06 **ab**	18	10.2 ± 2.32

Different letters indicate significant differences (p≤0.05); CAPcontrol: capacitation medium without supplementation; CAP1: capacitation medium supplemented with 1 µM of AntiOxBEN2; CAP10: capacitation medium supplemented with 10 µM of AntiOxBEN2; FERTcontrol: fertilization medium without supplementation; FERT1: fertilization medium supplemented with 1 µM of AntiOxBEN2; FERT10: fertilization medium supplemented with 10 µM of AntiOxBEN2.

5.4 Embryonic quality and developmental stages

Supplementation of the fertilization medium and/or semen capacitation medium showed no significant influence (p>0.05) on the quality of the produced embryos, i.e., the rate of embryos produced with grade 1 (Good), grade 2 (Fair) or grade 3 (Bad) (Table 4).

Table 4. Effect of oocytes and spermatozoa supplementation with AntiOxBEN2 on the quality of the produced embryos.

Groups	Good (%)	Fair (%)	Bad (%)
CAPcontrol x FERTcontrol	25.0 ± 8.18	50.0 ± 9.45	25.0 ± 8.18
CAPcontrol x FERT1	38.7 ± 8.75	25.8 ± 7.86	35.5 ± 8.59
CAPcontrol x FERT10	33.3 ± 12.17	40.0 ± 12.65	26.7 ± 11.42
CAP1 x FERTcontrol	40.7 ± 9.46	29.6 ± 8.79	29.6 ± 8.79
CAP1 x FERT1	26.3 ± 7.14	42.1 ± 8.01	31.6 ± 7.54
CAP10 x FERTcontrol	27.3 ± 9.50	36.4 ± 10.26	36.3 ± 10.26
CAP10 x FERT10	27.8 ± 10.56	44.4 ± 11.7	27.8 ± 10.56

CAPcontrol: capacitation medium without supplementation; CAP1: capacitation medium supplemented with 1 µM of AntiOxBEN2; CAP10: capacitation medium supplemented with 10 µM of AntiOxBEN2; FERTcontrol: fertilization medium without supplementation; FERT1: fertilization medium supplemented with 1 µM of AntiOxBEN2; FERT10: fertilization medium supplemented with 10 µM of AntiOxBEN2.

No differences ($p>0.05$) were observed in the different embryonic stages (morula, blastocysts or hatched) of produced embryos either at day 7 or day 9.

6. Discussion

Our results showed, for the first time, that the novel mitochondrial-directed antioxidant molecule, AntiOxBEN2, influenced both bovine sperm capacitation and fertilization processes improving early embryonic development, which confirms the positive effect of this molecule in the prevention of oxidative stress in bovine gametes. Indeed, the supplementation of the capacitation and fertilization media with AntiOxBEN2 presented a substantial influence on the cleavage rate of the embryos. Higher results were obtained when both media were supplemented with 1 µM AntiOxBEN2 compared to the untreated groups, except for the CAPcontrol x FERT1 group, where CAP media was not supplemented but also presented high cleavage rates.

These results clearly suggest that the AntiOxBEN2 exert its effect, not only on spermatozoa, but also on oocytes, which had already been previously described by Teixeira et al. (2020), although in this case this antioxidant molecule was only added to the maturation medium of the oocytes, and not to the fertilization medium, as in the present study. A beneficial effect of the AntiOxBEN2 resulting in higher cleavage rates was identified. Other substances, such as glutathione (Anchordfoquy et al., 2019), heparin and L-arginine (Santana et al., 2016) were previously tested in the fertilization medium, with the last two having beneficial effects on the cleavage rate and blastocyst production, while the first had no effect, at least on the tested concentrations.

In fact, antioxidants can act through different mechanisms, such as, hydrogen atom transfer, simple electron transfer and/or iron chelation (Pisoschi & Pop, 2015; Teixeira et al., 2017). Nowadays, several reports concerning the supplementation of extenders and cryopreservation media for gametes and embryos with antioxidants have arisen (Anchordoquy et al., 2019; Santana et al., 2016; Sapanidou et al., 2016; Tirpák et al., 2017), and while some have identified beneficial effects, others were unable of distinguish these effects. The ability of an antioxidant to perform its function, i.e. its ability to transfer a hydrogen atom or an electron to a stable free radical, is measured by inhibiting the absorbance of the radical, resulting from the deactivation of this radical by the antioxidant. Therefore, molecules with higher antioxidant power have a higher percentage of radical inhibition (Teixeira et al., 2017).

Phenolic antioxidants, such as AntiOxBEN2, can act in two ways regarding their ability to chelate iron and contribute to inhibit iron-mediated ROS generation. Comparing this capacity with that of a common mitochondrial antioxidant, such as MitoQ10, it was observed that AntiOxBEN2 presented the ability to chelate ferrous iron, a capacity that was superior to that of its precursor, and contrary to MitoQ10, this capacity can be explained by the introduction of the truncated spacer triphenylphosphonium (Teixeira et al., 2017). Even so, MitoQ10 was recently used to prevent oxidative stress during oocyte maturation and has shown positive results, increasing the rate of produced blastocysts (Marei et al., 2019). AntiOxBEN2 also has some capacity to prevent lipid mitochondrial peroxidation, exhibiting low toxicity profiles in different cell types and a significant decrease in redox potentials when compared with other antioxidants, such as antiOxBEN1 and protocatechuic acid (Teixeira et al., 2017), highlighting the huge potential of mitochondrial-directed antioxidants to improve ART outcomes.

The capacity of AntiOxBENs to be accumulated by energized mitochondria was successfully tested in isolated rat liver mitochondria (Teixeira et al., 2017). In the present study, a positive effect of both AntiOxBEN2 doses on mitochondria of bovine spermatozoa was identified. This compound increased the number of spermatozoa emitting a bright red-orange fluorescence after JC1 staining compared to control, without interfering on their vitality. Previous studies have identified JC1 dye as a powerful tool to differentiate the quality of the mitochondrial function in bovine sperm that could be related with fertility rates (Garner & Thomas, 1999; Nichi et al., 2017) as confirmed in the present study.

For this reason, our goal was to test this antioxidant specially developed to act in mitochondria (Teixeira et al., 2017), the main production site for ROS in germline (Aitken et al., 2016; Roth, 2018) in two crucial phases of *in vitro* artificial insemination processes: sperm capacitation and the moment of fertilization. Semen capacitation and acrosomal exocytosis are two crucial processes for semen quality and, consequently, for its capacity for fertilization, and are therefore closely related to male fertility (Fraser, 2010; Santana et al., 2016). It is also widely known that embryonic development is related to the quality of gametes (Krishnakumar et al., 2015). In *in vitro* methods, male and female gametes are exposed to high levels of ROS. These ROS reduce sperm motility, induce lipid peroxidation, generate DNA damage and as such sperm death. Moreover, they decrease the sperm-oocyte fusion capacity and delay embryonic development (Anchordoquy et al., 2019).

Comparing the two groups in which both media were supplemented, with 1 and 10 µM of AntiOxBEN2, the number of embryos produced at D7 at the concentration of 1 µM was more than double that of 10 µM. Therefore, although the cleavage rates of these two groups were similar, the concentration of 10 µM seemed already harmful in later phases. This harmful effect of the highest dose was also identified through the reduction of spermatozoa motility after the capacitation process. Both oocytes and spermatozoa are very sensitive to the negative impact of ROS but also of cytotoxicity that is reflected on their developmental potential (De Assis et al., 2015; Marques et al., 2018). Although the doses tested herein were not toxic to rat embryonic cardiomyoblasts, human neonatal dermal fibroblasts and hepatocellular carcinoma cells (Teixeira et al., 2017), our results clearly suggest the use of the lowest dosage when dealing with germplasm. According the authors, higher concentrations of this molecule, above those necessary to exert the antioxidant effect, showed a toxicity effect (Teixeira et al., 2017). Further studies should be performed to clearly identify the ideal dose to potentiate the AntiOxBEN2 beneficial effect on oocytes and spermatozoa.

By analyzing presented results, it was possible to assume that AntiOxBEN2 when supplemented to the fertilization medium was more effective than when added only to the sperm capacitation medium. This may be due to the fact that, when added during the fertilization process, it acts on both gametes and, in this case, it continues to act on both for twenty hours, until they were washed and placed in the SOF drops. Interestingly, the length of permanence of the AntiOxBEN2 on oocyte and zygote mitochondria is unknown and should be further investigated. The supplementation of the embryo culture media with this antioxidant molecule could probably also exert a beneficial effect as shown in the present work and by Teixeira and collaborators (2020).

Very significant effects of AntiOxBEN2 on the mitochondrial membrane potential of spermatozoa were also detected, in both tested concentrations, and compared to the control. At the concentration of 1 and 10 µM of AntiOxBEN2 higher values of bright red-orange fluorescence were obtained, which indicate higher membrane potentials (Garner & Thomas, 1999) that were associated with semen with the greatest potential for fertilization (Kasai et al., 2002; Trevizan et al., 2018) and this measurement presents itself as the most effective and sensitive to evaluate this capacity (Marchetti et al., 2002).

In the present study, when the antioxidant molecule was only added to the CAP medium, a beneficially proven effect on the fertilization process was not clearly identified, probably because the incubation time of semen with AntiOxBEN2 may have been short and/or the semen used may have good quality. Other authors (Sapanidou et al., 2016) showed promising results when longer incubation times of 120 and 240 minutes of sperm and antioxidants were conducted. Also, other published studies showed that men with semen of low quality (infertile) have higher levels of ROS than men with semen of high quality (fertile) (Martin-Hidalgo et al., 2019). Equally the bull sperm quality declines with advancing age, which was strongly associated with increased oxidative damage of both the spermatozoa plasma membrane and DNA (Trevizan et al., 2018). Infertility seems to be associated with high levels of ROS and oxidative stress, leading to greater cellular damage and decrease sperm viability. Thus, we can believe that the effect of AntiOxBEN2 would be more noticeable in semen with less viability since the ROS levels would expectedly be higher. This higher level of ROS is due, as previously described, to an imbalance between their production and the action of endogenous antioxidants (Valera-Alberni & Canto, 2018). In the future we should also test the antioxidant with semen of inferior quality than the one used in the present study.

Another hypothesis would be to add AntiOxBEN2 prior to the semen cryopreservation, to prevent damage caused by the ROS produced in the freeze-thaw processes. Similar tests with another antioxidant, ascorbic acid, have already been carried out and have had positive effects on sperm quality (Hu et al., 2010).

7. Conclusion

Due to the rapid development of assisted reproduction techniques it became essential to develop molecules capable of effectively combating ROS produced *in vitro* and infertility problems. For this reason, the novel mitochondrial-directed antioxidant, AntiOxBEN2 was developed and then tested in this study. Our data confirm that AntiOxBEN2 has beneficial effects on the mitochondrial membrane potential and consequently on sperm quality, when added to the capacitation medium. Moreover, it was also possible to observe an improvement of cleavage rates and the number of D7 produced embryos, when supplemented to the fertilization medium. These results are promising and indicate that further research are required, namely using AntiOxBEN2 at other concentrations, between 1 and 10 µM, longer incubation times for the spermatozoa and its introduction in different stages of the *in vitro* embryo production process. Other strategy would be to test AntiOxBEN2 during semen cryopreservation. In conclusion, the investigation for the benefits of AntiOxBEN2 supplementation on sperm quality requires further studies.

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