ESCOLA UNIVERSITÁRIA VASCO DA GAMA

MESTRADO INTEGRADO EM MEDICINA VETERINÁRIA

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Maria Manuela Martins Silvério Pereira Jorge

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Maria Manuela Martins Silvério Pereira Jorge
Aluna do Mestrado Integrado em Medicina Veterinária

Constituição do Júri

Presidente do Júri
Professor Doutor Hugo Corte-Real Vilhena
Arguente
Professor Doutor Pedro Nuno d'Almeida Monteirinho Pinto Bravo
Orientador
Professora Doutora Sofia Alexandra Giestas Cancela Duarte

Orientador Interno
Professora Doutora Sofia Alexandra Giestas Cancela Duarte

Orientador(es) Externo(s)
Professora Doutora Rosa Lino Neto Pereira
Professor Doutor José Carlos Santos Teixeira

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"Torna-te um perfeito estranho para a tua vontade e prossegue"
(J.Tolentino Mendonça)

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List of acronyms and abbreviations

ALH: amplitude of lateral head displacement
AMPc: cyclic adenosine monophosphate
ART: assisted reproductive techniques
ATP: adenosine triphosphate
BCF: beat cross frequency
BL: blastocysts
BSA: bovine serum albumin
CAP: capacitation medium
CASA: Computer-Assisted Sperm Analysis
COC: cumulus oocyte complexes
DNA: deoxyribonucleic acid
EBL: expanded blastocysts
FCS: fetal calf serum
FERT: fertilization medium
HBL: hatched blastocysts
IETS: International Embryo Technology Society
IVF: *in vitro* fertilization
LIN: linearity
M: morulae
MMP: mitochondrial membrane potential
NOX: nicotinamide adenine dinucleotide phosphate oxidase
OCR: oxygen consumption rate
OXPHOS: oxidative phosphorylation
PBS: phosphate buffered saline
ROS: reactive oxygen species
SOF: synthetic oviductal fluid
STR: straightness
TCM: tissue culture medium
UA: urolithin A
VAP: average path velocity
VCL: curvilinear velocity
VSL: straight-line velocity
YBL: young blastocyst

animals

MDPI

Article

Effect of Urolithin A on Bovine Sperm Capacitation and *in vitro* Fertilization

Manuela Jorge[1,2], Filipa C. Ferreira[1,3], Carla C. Marques[1], Maria C. Batista[1], José Teixeira[4], Paulo J. Oliveira[4], Sofia C. Duarte[2,5], Rosa M. L. N. Pereira[1,2,6*]

1 Unit of Biotechnology and Genetic Resources Unit, National Institute of Agrarian and Veterinary Research, Quinta da Fonte Boa, 2005-048 Vale de Santarém, Portugal; manuela.pjorge@gmail.com (M.J.); filipa.ferreira@iniav.pt (F.C.F.); carla.marques@iniav.pt (C. C. M.); conceicao.batista@iniav.pt (M.C.B.); rosa.linoneto@iniav.pt (R. M. L. N. P.);
2 Department of Veterinary Sciences Research Centre, Vasco da Gama University School, 3020-210, Lordemão University Campus, Coimbra, Portugal; sofia.duarte@euvg.pt (S.C.D.);
3 GeoBioTec - Faculty of Sciences and Technology, New University of Lisbon, Portugal;
4 CNC-Centre for Neurosciences and Cell Biology, University of Coimbra, Portugal; jcsteixeira8@gmail.com (J.T.); pauloliv@cnc.uc.pt (P.J.O.);
5 LAQV, REQUIMTE, Laboratory of Bromatology and Pharmacognosy, Faculty of Pharmacy, University of Coimbra, Polo III, Coimbra, Portugal;
6 CIISA, Faculty of Veterinary Medicine, University of Lisbon, and Associated Laboratory for Animal and Veterinary Science (AL4AnimalS); Av. da Universidade Técnica.
* Correspondence: rosanlp@gmail.com or rosa.linoneto@iniav.pt; Tel.: +351-00345767300

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Simple Summary: Sperm cells are specialized cells highly susceptible to different kind of damage impairing the success of *in vitro* fertilization and, consequently, of assisted reproductive techniques (ART). Oxidative stress plays a significant role in this context, causing DNA injury, reduced motility, and compromising the fertilization ability of sperm. The use of antioxidants aims to mitigate the oxidative stress, protect sperm cells from damage, and enhance their fertilization potential. Urolithin A (UA) is a natural compound with proven anti-aging and antioxidant effects in various cells, but its effect on bovine sperm has not been tested yet. The objective of this study was to investigate the impact of UA on bovine sperm function and fertilization rate, thus contributing to animal reproductive development. The results showed that UA improved sperm motility quality and reduced oxidative stress levels in a dose-dependent manner. The use of UA as an antioxidant therapy may have a significant impact on the advancement of reproductive biotechnologies.

Abstract: Reactive oxygen species (ROS) play a critical role in the functional competence of sperm cells. Conversely, excessive generation of ROS can impair sperm function, including their ability for fertilization. Urolithin A (UA), a natural metabolite with anti-aging and antioxidant properties, was investigated for the first time in bovine sperm cells in the present study. Firstly, different doses of UA (0, 1, and 10 μM; 8-16 sessions) were used during the capacitation process of frozen-thawed bovine sperm. Sperm motility was assessed using optical microscopy and CASA. Sperm vitality (eosin-nigrosin), ROS and ATP levels, as well as mitochondrial membrane potential (JC1) and oxygen consumption were evaluated. A second experiment to test the effect of different doses of UA (0, 1, and 10 μM; 6 sessions) in both the capacitation medium, as above, and the fertilization medium, was also implemented. The embryonic development and quality were evaluated. UA at a concentration of 1 μM significantly improved sperm movement quality ($p < 0.025$). There was a trend towards an increase in the oxygen consumption rate (OCR) of capacitated sperm with 1 μM and 10 μM UA supplementation. Moreover, an increase in ATP levels ($p < 0.01$) was observed, accompanied by a reduction in ROS levels at the higher UA concentration. These results suggest that UA may enhance spermatozoa mitochondrial function. UA almost doubled the number of produced embryos without significant differences in quality. Presented results further support previous findings indicating the potential therapeutic value of UA for addressing reproductive sub/infertility problems and improving ART results.

Keywords: Urolithin A; spermatozoa; oocyte; ROS; mitochondria; ART; reproductive; biotechnologies

1. Introduction

Urolithins are natural compounds produced by the gut microbiota from ellagic acid and ellagitannins found in fruits such as pomegranates, strawberries, and nuts. Urolithin A (UA), also known as 3,8-Dihydroxyurolithin ($C_{13}H_8O_4$) (Figure 1), is one of the most important compounds in this group [1]. Recently several tests were conducted to investigate the activity and safety of UA isoforms. Studies have shown that UA has a good bioaccessibility and safety profile, with potential to modulate oxidative stress and recover tissue damage through different mechanisms [2]–[7].

The impact of UA on the prevention of aging [8] and disease processes was postulated, as it enhances cellular health by increasing mitophagy and mitochondrial function [9]. Mitochondria are organelles that have been described as the "Powerhouses of the Cells" but are also responsible for producing ROS [10]. The overproduction of ROS, together with a failure of balance in the body's antioxidant enzyme systems, can result in a situation of oxidative stress that is a determining factor in the etiology of many pathologies, including the reproductive ones [11]–[13]. Indeed, dysfunctional mitochondria cannot provide the energy needed by cells, particularly those with high energy consumption, such as spermatozoa (spz) [11].

Conversely, the oxidative stress has a critical role in the life and death of specialized mammalian spz. These cells produce reactive oxygen species (ROS) from three different sources, which include sperm mitochondria, cytosolic L-amino acid oxidases, and plasma membrane nicotinamide adenine dinucleotide phosphate oxidases (NOXs). Physiological levels of ROS promote sperm capacitation, while excess ROS exposure, or depleted antioxidant defenses, yields a state of oxidative stress, which disrupts their fertilizing capacity [14], [15]. ROS-induced spz changes include the activation of extracellular signal regulated kinase-like proteins, up-regulation of tyrosine phosphorylation in the tail, and the induction of sterol oxidation, which are triggered by the stimulation of a cyclic adenosine monophosphate (AMPc)/protein kinase A phosphorylation cascade [14]. However, excessive generation of ROS can lead to lipid peroxidation, which disrupts membrane characteristics that are essential for maintaining sperm function, including their ability to fertilize oocytes [8], [9]. Furthermore, lipid aldehydes produced due to lipid peroxidation can bind to proteins in the mitochondrial electron transport chain, triggering a cycle of further ROS generation. Ultimately, the high levels of oxidative stress can damage the DNA in the spermatozoon nucleus [14]. Guanine is the most readily oxidized of the four DNA bases being converted to the mutagenic form 8-hydroxy-deoxyguanosine (8-OHdG) [14], [15]. Overall, this evidence indicates sperm sensitivity to this type of stress that can affects every aspect of the reproductive process from gametogenesis to the birth of healthy offspring [14], [16].

Sperm cells require a balance of oxidants and antioxidants to function properly, and sperm preservation methods can cause oxidative stress [17]. There are still challenges associated with semen cryopreservation and the harmful effects of oxidative stress on sperm quality. Spermatozoa mitochondria generate ROS during oxidative phosphorylation, which plays a vital role in signaling pathways essential for sperm fertilizing ability. During cryopreservation, excessive ROS production leads to mitochondrial dysfunction, which causes a decrease in mitochondrial membrane potential and altered sperm functions. The processes of osmotic and thermal stress, as well as intracellular ice crystal formation, also contribute to this excessive ROS production. These events potentiate cryoprotectant-induced calcium overload in the mitochondrial matrix, triggering the opening

of mitochondrial permeability transition pores and resulting in the release of pro-apoptotic factors from the mitochondria, initiating an apoptotic cascade [18].

According to Fonseca and col. [8], UA was tested for the first time in animal reproduction, namely in bovine oocytes subjected to *in vitro* maturation. In this cited study, a model for aging the female gamete was implemented and the negative effect of oocyte aging on its developmental competence was confirmed. The model proved to be very efficient and useful regarding reproductive diseases, gamete ageing and consequent decrease in fertility. UA supplementation to the maturation medium was established as a new therapeutic approach in assisted reproductive biotechnologies, as UA could prevent or delay female gamete ageing. Moreover, UA has improved blastocyst formation and fertility outcomes. However, UA has never been tested on bovine or other species spermatozoa.

The present study intended to evaluate the impact of UA on bovine sperm function, fertilization, and embryo production rates. This approach makes this study innovative, which may contribute to the development of therapies for male sub/infertility and reproductive biotechnologies, an area that is expanding but lacks research.

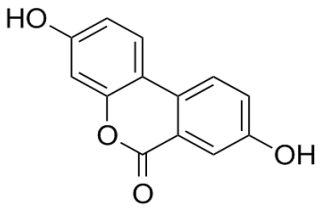

Figure 1. Chemical structure of the Urolithin A (UA; modified from https://pubchem.ncbi.nlm.nih.gov/compound/5488186).

2. Materials and Methods

2.1. Experimental Design

This study was approved by the Ethical Commission of EUVG (nº 15/2022) with the following objectives:

a) to test the effect of different concentrations of UA (0, 1 and 10 μM; 8 - 16 replicates) in the capacitation process of frozen-thawed bovine spz on their morphology, motility, viability, ROS production and mitochondrial quality. The process of maturation in the capacitation medium (CAP; CAP0 - 0 μM of UA, CAP1 – 1 μM of UA and CAP10 – 10 μM of UA) was carried out by the swim-up method for one hour. Then, spz were observed using optical microscopy to characterize them in terms of morphology, motility, and vitality. Additionally, through Computer Assisted Sperm Analysis (CASA) parameters such as total motility, progressive motility, curvilinear velocity parameters, were evaluated. The Seahorse XFe96 technique allowed the quantification of mitochondrial respiration, to achieve a better understanding of cellular metabolism and mitochondrial function. Sperm ATP and ROS levels were also measured. For this purpose, spz from two bulls of proven fertility (pools of 4 straws from ejaculates from two collection days), were used.

b) to test the effect of different doses of UA in both the capacitation medium, as above, and the fertilization medium (Fert0, Fert1 and Fert10), on sperm fertilizing ability and subsequent early embryo development (6 sessions). For this purpose, bovine ovaries (n = 642) were collected in a local abattoir, immediately after slaughter, in 6 sessions, for oocyte collection (n = 2200) at the Embryology Laboratory of *Instituto Nacional de Investigação Agrária e Veterinária (INIAV).* After oocyte selection, oocytes were matured for 22 hours

and transferred to UA supplemented fertilization medium. Then mature oocytes were inseminated with the capacitated spz, with UA at the concentration of 0 (control), 1 and 10 μm, totalizing seven groups (Table 1). After 22 h of co-incubation, presumptive zygotes were transferred to culture medium and afterwards the cleavage rate, the blastocyst rates on days 7 (D7) and 8 (D8), and the quality of the embryos were evaluated.

Table 1. Experimental design of the second experiment to study the effect of UA in the process of the capacitation of bovine spermatozoa and fertilization of oocytes. UA was added to the capacitation medium (CAP) and/or the fertilization medium (FERT) at the concentration of 0 (control), 1 and 10 μM originating seven groups.

CAP	FERT	Groups
CAPcontrol	FERTcontrol	CAPcontrol x FERTcontrol
	FERT1	CAPcontrol x FERT1
	FERT10	CAPcontrol x FERT10
CAP1	FERTcontrol	CAP1 x FERTcontrol
	FERT1	CAP1 x FERT1
CAP10	FERTcontrol	CAP10 x FERTcontrol
	FERT10	CAP10 x FERT10

2.2. Oocyte Preparation

At an abattoir in Santarém, bovine ovaries were collected to thermos bottles with a solution of Dulbecco's Phosphate Buffered Saline (PBS) at 37 ºC and transported to the laboratory. The PBS was previously supplemented with 0.15 % (w/v) bovine serum albumin (BSA) and 0.05 mg mL^{-1} of kanamycin. At the laboratory, the ovaries were washed with the same solution and the cumulus oocyte complexes (COCs) were obtained by post-mortem follicular aspiration, from a heterogeneous pool of antral follicles, with 2 to 8 mm in size [19]. Then, the collected COCs presenting more than three layers of cumulus cells and homogenous ooplasm were selected for maturation in an incubator at 38,8 ºC, with a maximum humidity atmosphere and 5 % CO_2, for 22 hours. Maturation medium consisted of TCM199 without Hepes, with 10 % fetal bovine serum (FCS), 10 ng ml^{-1} epidermal growth factor (EGF), 0.1 ml sodium pyruvate and antibiotic (4 μl ml^{-1} gentamicin) [20].

2.3. Spermatozoa Preparation and Analysis

Bovine semen from two bulls of proven fertility was thawed (4 straws for each session, 6-8 sessions) at 37 ºC for 30 seconds. Then, it was carefully deposited in equal doses at the bottom of the tubes containing the CAP consisting of modified Tyrode´s medium without calcium supplemented with 2.4 mM of caffeine [21], that was previously prepared and supplemented with different concentrations of Urolithin A (0; 1 and 10 μM). Afterwards, spermatozoa were placed in the incubator for 1 hour in an atmosphere of 5 % CO_2, at 38.8 ºC with maximum humidity [19], [22].

The *in vitro* capacitation process (swim up) occurs during this period when the most active spz migrate to the surface of the liquid. The supernatant was collected and centrifuged at 2300 rpm for 10 minutes. The supernatant was again aspirated and discarded. The motility and concentration of spz were immediately evaluated.

2.3.1. Evaluation of Spermatozoa Movement

After the swim up process, semen motility was evaluated to assess their quality, through CASA assay. This assay is a reliable tool that provides precise and accurate information on kinematic parameters [23]. The parameters evaluated were total and progressive motility (%); curvilinear velocity (VCL, μm s^{-1}); average path velocity (VAP, μm s^{-1}); static (%), slow, medium and rapid motile spz (n); straight-line velocity (VSL, μ ms^{-1}); amplitude of lateral head displacement (ALH, μm); linearity; straightness; wobble VAP/VCL and beat cross frequency (BCF, Hz).

2.3.2. Evaluation of Spermatozoa Vitality and Morphology

Spermatozoa vitality and morphology were evaluated using the eosin-nigrosine dye, following a procedure adapted from Pereira and col. [24]. To conduct the analysis, equal amounts of capacitated sperm samples and the dye were mixed on a microscope slide. A cover slip was used to smear the mixture, which was then air-dried and directly examined. At a magnification of 1000× under oil immersion, at least 100 sperm cells were evaluated in each sample. The cells that remained unstained were classified as live, while those that showed any pink or red coloration were classified as dead. Moreover, the presence of any morphological abnormalities in the sperm's tail, midpiece, and head was also examined.

2.3.3. Evaluation of Mitochondrial Membrane Potential

The mitochondrial membrane potential (MMP) is a measure of mitochondrial activity and was assessed in this study using a fluorescent probe called JC-1 (Invitrogen, UK). JC-1 can differentiate between fully functional midpiece mitochondria, which exhibit orange/red fluorescence, and cells with lower inner mitochondrial membrane potential, which exhibit green fluorescence. Spermatozoa were subjected to different treatments (CAPcontrol, CAP1, and CAP10) during the swim-up process and then incubated with JC-1 in a solution containing Hanks, HEPES, and BSA for 30 minutes at 38.8 °C and 5 % CO in humidified air, in the absence of light. After incubation, sperm samples were placed on warmed glass microscope slides, with coverslips, and examined using a fluorescence microscope (Olympus BX51) with a blue fluorescence filter (BP 470-490, objective UPlanFI 20×/0.50). One hundred spermatozoa in each sample were examined for staining patterns.

2.3.4. Intracellular ATP Levels

To determine intracellular ATP levels, (2 x 10^6) swimmed-up spermatozoa from different treatments were seeded in a 96-well plate with a white opaque bottom and cultured in 50 μL of medium. The CellTiter-Glo Luminescent Cell Viability Assay (Promega, WI, USA) was used to measure ATP levels as per the manufacturer's protocol. In brief, 50 μL of CellTiter-Glo Reagent (CellTiter-Glo Buffer + CellTiter-Glo Substrate) was added to the cells and the mixture was shaken on an orbital shaker for 2 minutes to induce cell lysis. The luminescence signal was then monitored after incubation at 22 °C for 10 minutes, using a Cytation 3 reader (Biotek Instruments, Winooski, VT, USA).

2.3.5. Evaluation of Intracellular Oxidative Stress

To evaluate the intracellular oxidative stress, (2 x 10^6) swimmed-up spz were seeded in 96-well plates after being treated with CAPcontrol, CAP1, and CAP10. The report molecule CM-H2DCFDA (5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester) (Life Technologies, Invitrogen) was used for this purpose. The cells were loaded with 5 μM of CM-H2DCFDA in assay buffer, and fluorescence signals were measured with 520 nm excitation and 620 nm emission wavelengths during 30 minutes at 38.5 °C, to determine the CM-H2DCFDA oxidation rate in a microplate reader (Cytation 3; BioTek Instruments, Winooski, VT, USA). The sulforhodamine B (SRB) assay was used

to normalize the results for cell mass content after cell fixation with trichloroacetic acid (TCA) 60 % (w/v).

2.3.6. Evaluation of Cell Mass

To evaluate the cell mass content, the sulforhodamine B (SRB) assay was used. Briefly, fixation solution was discarded, and the plates were dried in an oven at 37 ºC. One hundred and fifty microliters of 0.05 % SRB in 1 % acetic acid solution were added and incubated at 37 °C for 1 h. The wells were then washed with 1 % acetic acid in water and dried. Then, 100 μL of Tris (pH 10) was added, and the plates were stirred for 15 min. The optical density was measured at 540 nm in Biotek Cytation 3 reader (Biotek Instruments, Winooski, VT, USA).

2.3.7. Analysis of Cellular Oxygen Consumption Rate

Following capacitation, the spz (CAPcontrol, CAP1, and CAP10 groups) were separated by centrifugation and added to a pre-coated 96-well plate containing 0.5 mg mL^{-1} Concanavalin A (ConA) in a modified synthetic oviductal fluid (SOF) medium [16]. The concentration used was 1 x 10^6 cells per well, with 8 wells assigned for each group and bull (16 replicates). Cellular oxygen consumption was measured at 37 ºC utilizing a Seahorse XFe96 Extracellular Flux Analyzer (Agilent, Germany) [25]. An XFe96 sensor cartridge was placed in a 96-well calibration plate containing 200 μL $well^{-1}$ calibration buffer to hydrate at 37 ºC, a day prior to the experiment. The OCR measurements were initiated by injecting 1 μM rotenone and 1 μM antimycin A into reagent delivery port A. Then, 25 μL of compounds were loaded into the ports of each well in the XFe96 sensor cartridge, and both the sensor cartridge and the calibration plate were loaded into the XFe96 Extracellular Flux Analyzer for calibration. Once the calibration was completed, the calibration plate was replaced with the study plate. Three OCR baseline rate measurements were performed using a 3-minute mix, 5-minute measure cycle. The compounds were pneumatically injected by the XFe96 Analyzer into each well, mixed, and OCR measurements were taken using a 3-minute mix, 5-minute measure cycle. Finally, the results were analyzed using Software VersionWave Desktop 2.6.

2.4. Oocyte Insemination, Embryo Culture and Development

In this study, spermatozoa (2 x 10^6 spz mL^{-1}) were introduced into droplets of fertilization medium (40 μL), each containing 10 mature oocytes. The *in vitro* fertilization medium was composed of modified Tyrode's medium enriched with 5.4 USP mL^{-1} of heparin, 10 mM of penicillamine, 20 mM of hypotaurine, and 0.25 mM of epinephrine, as described by Fonseca and col. [8]. The medium was supplemented with Urolithin A (0; 1; e 10 μM). The spz and oocytes were co-incubated for 20 h at 38.8 ºC and 5 % CO_2 in humidified air. Then denuded presumptive zygotes were cultured in 25 μL droplets of SOF supplemented with minimum essential medium (MEM) non-essential amino acid solution, basal medium eagle (BME) amino acids solution, glutathione, and BSA. Embryo culture was maintained at 38.8 ºC in a humidified atmosphere with, 5 % CO_2, 5 % O_2 and 90 % N_2. Cleavage rates were assessed at 48 hours post-insemination, and embryo development continued for 10 days in SOF + BSA supplemented with 10 % fetal calf serum (FCS) (IVF = day 0) under the same conditions.

The assessment of embryo development included cleavage rates as a proportion of cleaved embryos and inseminated oocytes. Days 7 and 8 embryo (morula and blastocysts) developmental rates are used as a proportion of morula and blastocysts and cleaved embryos [19]. In addition, on D7/D8, the quality of embryonic development was assessed and classified based on International Embryo Technology Society (IETS) guidelines as good, fair, or bad [26]. The different stages of embryonic development at D7 and D9 were

identified as morulae (M), young blastocysts (YBL), blastocysts (BL), expanded blastocysts (EBL), and hatched blastocysts (HBL).

2.5. Statistical Analysis

To analyze the data from sperm parameters, the MIXED procedure of the Statistical Analysis Systems Institute (SAS Inst., Inc., Cary, NC, USA) was used. This procedure employed a mixed linear model that considered the treatment (CAP) and bull as fixed effects, allowing the calculation and comparison of means for each treatment. Sessions were considered a random effect. 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 (CAP x FERT) and session as fixed and random effects, respectively. Comparisons between groups were performed using the PDIFF test.

GraphPad Prism 8.0 software (GraphPad Software, Inc., San Diego, CA, USA) was used for data analysis of intracellular oxidative stress, ATP levels, and oxygen consumption. The results were presented as means ± standard error of the mean (SEM) for the indicated number of experiments. One-way ANOVA analysis, followed by Dunnet's post-test, was used to compare groups with one independent variable and determine statistical significance. A significance level of $p \leq 0.05$ was used to determine statistical differences in the values.

3. Results

3.1. Effect of UA in Spermatozoa Kinematic Parameters

Results obtained with the supplementation of the capacitation medium with UA were depicted in Table 2.

Table 2. Effect* of supplementation of the capacitation medium with Urolithin A (UA) on the kinematic parameters of semen using Computer Assisted Sperm Analysis (CASA, 8 replicates).

Kinematic Parameters	Groups		
	CAPcontrol	CAP1	CAP10
Total Motility (%)	65.9 ± 5.32^{a}	63.5 ± 5.19^{ab}	56.1 ± 5.34^{b}
Progressive Motility (%)	32.4 ± 5.75^{ab}	34.2 ± 5.66^{a}	23.1 ± 5.76^{b}
VCL (μms^{-1})	70.9 ± 6.54	68.0 ± 6.32	65.9 ± 6.55
VAP (μms^{-1})	41.3 ± 5.66	45.7 ± 5.52	43.5 ± 5.67
Static spz (n)	33.3 ± 5.36^{b}	35.7 ± 5.21^{ab}	43.3 ± 5.36^{a}
Slow motile spz (n)	2.7 ± 0.75	3.0 ± 0.72	2.2 ± 0.75
Medium motile spz (n)	20.9 ± 6.05	26.4 ± 6.05	2.1 ± 1.65
Rapid motile spz (n)	12.0 ± 2.27^{a}	8.3 ± 2.13^{ab}	5.9 ± 2.27^{b}
VSL (μms^{-1})	35.1 ± 5.77	43.6 ± 5.61	38.1 ± 5.78
ALH (μm)	2.7 ± 0.24	2.1 ± 0.23	2.2 ± 0.24
Linearity	47.8 ± 3.44^{b}	60.8 ± 3.21^{a}	56.4 ± 3.43^{ab}
Straightness	78.5± 3.32^{b}	90.7 ± 3.03^{a}	82.8 ± 3.30^{ab}
Wobble VAP/VCL	56.6 ± 2.77^{b}	65.2 ± 2.59^{a}	63.1 ± 2.76^{ab}
BCF (Hz)	6.89 ± 0.51	7.3 ± 0.47	6.6 ± 0.51

*The data are represented as estimated mean ± standard error of the mean. Different letters indicate significant differences ($p \leq 0.05$). VCL: curvilinear velocity; VAP: average path velocity; spz: spermatozoa; VSL: straight-line velocity; ALH: lateral head displacement amplitude; BCF: beat cross frequency. CAP 0: capacitation medium without supplement (control); CAP 1: capacitation medium supplemented with 1 μM UA; CAP 10: capacitation medium supplemented with 10 μM UA.

Urolithin A at a concentration of 1 μM improved ($p \leq 0.025$) sperm movement quality evaluated through CASA: linearity ($p = 0.0016$), straightness ($p = 0.0065$) and wobble ($p = 0.0094$) when compared to the control group.

Urolithin A at a concentration of 10 μM impaired total ($p = 0.0124$) and progressive ($p = 0.0108$) motility when compared to CAPcontrol or CAP1 groups, respectively. Moreover, higher number of static spermatozoa ($p = 0.0105$) and lower number of the rapid ones ($p = 0.0137$) were evaluated than in control (Table 2).

Significant differences ($p \leq 0.05$) in sperm quality were identified between bulls A and B. The better kinematic features were observed in bull A (Total Motility, VCL, ALH and Rapid motile spz).

3.2. Effect of UA on Semen Vitality, Abnormalities, and Mitochondrial Membrane Potential

Sperm quality can be determined by measuring three important parameters: motility, morphology, and vitality [27]. Furthermore, a deepen analysis of this quality can be achieved through accurate measures of sperm mitochondrial functionality [16], [20]. After capacitation with UA, the sperm samples were evaluated for concentration, vitality, as well as for the presence of head, intermediate piece, and tail anomalies. Mitochondrial membrane potential was also assessed. The results obtained showed an effect ($p = 0.0016$) of UA at the concentration of 10 μM increasing head defects of spermatozoa when compared to the control group (Table 3). No significant differences were observed in the remaining morphological and vitality parameters. However, differences ($p \leq 0.05$) were identified between bulls A and B. In terms of vitality features, bull A showed increased vitality (bull A = 52.9 % and bull B = 26.2 %). Fewer anomalies (head defect: bull A = 6.9 %; bull B = 10.7 %; intermediate piece defect: bull A = 3.2 %, bull B = 5.3 %) were observed in bull A.

Mitochondrial function was evaluated by measuring the mitochondrial membrane potential (MMP). Depolarized mitochondria, characterized by a low MMP, predominantly exhibit green fluorescence due to the presence of JC-1 monomers. On the other hand, hyperpolarized mitochondria with a high MMP accumulate JC-1 aggregates, emitting red fluorescence [28]. Results of MMP were represented in Table 3 but there were no significant effect of UA or differences between bulls ($p \geq 0.05$).

Table 3. Effect* of supplementation of the capacitation medium with UA on sperm concentration, vitality, morphology, and mitochondrial membrane potential (8 replicates).

Parameters	Groups		
	CAPcontrol	**CAP1**	**CAP10**
Concentration (x10^6 spz mL^{-1})	36.7 ± 6.23	36.4 ± 6.23	40.9 ± 6.23
Vitality (%)	39.2 ± 3.38	42.8 ± 3.16	36.6 ± 3.16
Head defect (%)	7.2 ± 1.04^{b}	7.9 ± 0.97^{ab}	11.2 ± 0.97^{a}
Interm.piece defect (%)	3.9 ± 0.56	4.2 ± 0.51	4.8 ± 0.51
Tail defect (%)	2.9 ± 0.61	3.2 ± 0.58	3.1 ± 0.58
Red-orange fluorescence (%)	83.4 ± 3.03	83.7 ± 3.03	84.2 ± 2.52
Green fluorescence (%)	16.6 ± 3.03	16.3 ± 3.03	15.8 ± 2.52
Ratio (%)	6.8 ± 3.96	9.3 ± 3.96	6.7 ± 3.38

*The data is presented as the mean value ± standard error of the mean. CAPcontrol: capacitation medium without supplementation; CAP1: capacitation medium supplemented with 1 μM of UA; CAP10: capacitation medium supplemented with 10 μM of UA. Different letters indicate significant differences ($p \leq 0.05$).

3.3. Effect of UA on spermatozoa Oxygen Consumption Rate, Cellular Oxidative Stress and Intracellular ATP Levels

The OCR is a crucial indicator of mitochondrial activity, specifically the electron transport rate [28], [29]. The Seahorse XF-96 Extracellular Flux Analyzer was used to measure the impact of UA on the mitochondrial oxygen consumption of spermatozoa after capacitation. Results showed that UA tended to increase basal OCR regardless of the dose (control = 32.31 ± 4.19 pmol min^{-1} 1M spz^{-1}; CAP1 = 46.01 ± 7.35 pmol min^{-1} 1M spz^{-1}; and CAP10 = 46.99 ± 9.45 $pmol^{-1}$ min^{-1} 1M spz^{-1}) (Figure 2**A**).

Oxidative stress impacts male and female germ lines, with moderate ROS levels benefiting sperm function and the enhancement of sperm capacitation [12]. In the present study, the impact of the UA on oxidative stress in spermatozoa was evaluated after capacitation, using a fluorescent reporter molecule (CM-H2DCFDA). In this case, results showed that UA tended to decrease ROS levels in a dose-dependent manner (control = 100.0 ± 31.02; CAP1 = 81.14 ± 36.80; and CAP10 = 26.12 ± 4.77) (Figure 2**B**).

Sperm motility hinges on the energy obtained through ATP hydrolysis, which is facilitated by mitochondria located in the sperm's intermediate piece. Sperm cells acquire their energy from two main pathways: OXPHOS and glycolysis. In the case of bovine sperm, OXPHOS appears to be favored over glycolysis [28]-[30]. After capacitation, UA increased ATP levels in a dose dependent manner ($p \leq 0.01$) (Figure 2**C**), when compared to control group (control = 100.0 ± 26.6; CAP1 = 104.1 ± 6.9; and CAP10 = 304.4 ± 36.9).

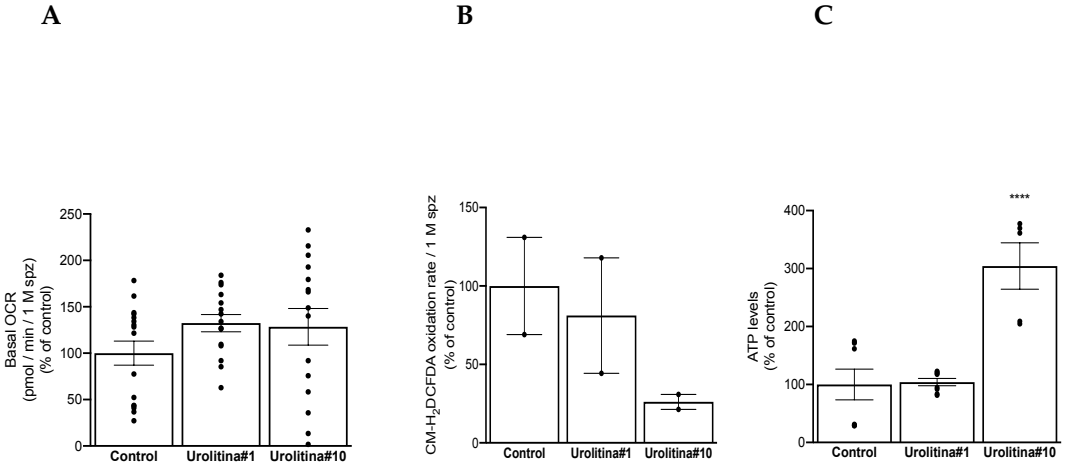

Figure 2. Effect of Urolithin A supplementation (0, 1, and 10 μM), on mitochondrial oxygen consumption rate, ATP levels, and ROS levels during the capacitation process of bovine sperm. (**A**) Basal oxygen consumption rate (OCR) was analyzed using the Seahorse XFe96 Extracellular Flux Analyzer. Data are means ± standard error of the mean of 16 replicates (8 for each bull), and results are expressed in pmol O_2^{-1} min^{-1} cell $mass^{-1}$. (**B**) Mean fluorescence signal of the cellular oxidation product CM-H2DCFDA in bovine sperm in the presence or absence of UA supplementation (1 and 10 μM) during the capacitation process. Data are means ± standard error of the mean of 8 replicates, and results are expressed as CM-H2DCFDA fluorescence cell mass of sperm. (**C**) Intracellular ATP levels in bovine sperm in the presence or absence of UA supplementation (1 and 10 μM) during the capacitation process. Data are means ± standard error of the mean of 8 replicates, and results are expressed as cell mass ATP levels per sperm. **** Indicates differences at $p \leq 0.01$; Control: capacitation medium without supplementation; Urolithin#1: capacitation medium supplemented with 1 μM of UA; Urolithin#10: capacitation medium supplemented with 10 μM of UA.

3.4. Effect of UA on Embryo Production Rates and Quality

The results obtained after the supplementation of UA to the CAP medium and/or to the FERT medium were represented in Table 4 and Figure 3.

Table 4. Effect* of different concentrations of UA during capacitation and/or fertilization processes on cleavage and embryo production rates (6 sessions).

Groups	Inseminated Oocytes (n)	Cleavage (%)	D7 Embryos	
			(n)	(%)
CAPcontrol x FERTcontrol	254	66.2 ± 3.00	15	9.0 ± 2.30
CAPcontrol x FERT1	265	70.6 ± 2.83	27	15.8 ± 2.85
CAPcontrol x FERT10	323	68.7 ± 2.65	23	11.3 ± 2.33
CAP1 x FERTcontrol	256	72.1 ± 2.84	25	15.0 ± 2.92
CAP1 x FERT1	319	67.7 ± 2.69	27	13.9 ± 2.65
CAP10 x FERTcontrol	214	75.8 ± 2.94	25	16.7 ± 3.11
CAP10 x FERT10	264	66.7 ± 2.94	19	12.5 ± 2.80

*The data is presented as the mean value ± standard error of the mean. CAPcontrol: capacitation medium without supplementation; CAP1: capacitation medium supplemented with 1 µM of UA; CAP10: capacitation medium supplemented with 10 µM of UA. FERTcontrol: fertilization medium without supplementation; FERT1: fertilization medium supplemented with 1 µM of UA; FERT10: fertilization medium supplemented with 10 µM of UA

This supplementation did not present a significant influence on the embryo cleavage ($p = 0.2890$) (Table 4) and production rate at D7/8 ($p \geq 0.05$, Figure 3). Nevertheless, the number of produced embryos almost double after UA supplementation, exception made for the CAP10 x FERT10 group (Figure 3).

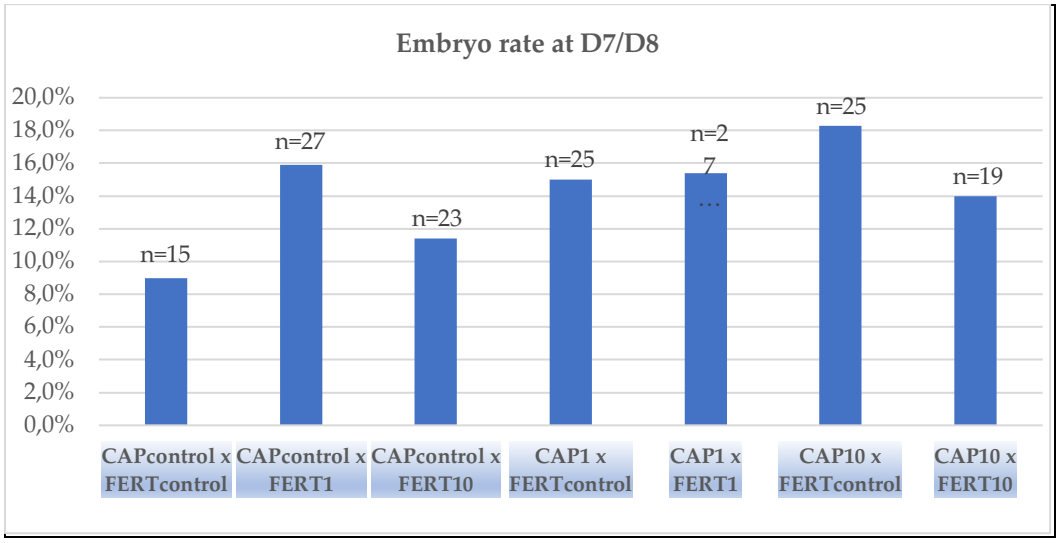

Figure 3. Effects of different concentrations of UA during capacitation and/or fertilization processes on embryo production rates at day 7/8 (mean ± SEM, 6 sessions).

No significant differences ($p \geq 0.05$) were observed in the quality rates of the produced embryos (Table 5).

Table 5. Effect* of UA supplementation during capacitation and/or fertilization processes on the quality of Day 7/8 embryos (mean ± SEM, 6 sessions). The embryo quality was classified based on morphological criteria, by International Embryo Technology Society (IETS) guidelines, as excellent/good (Grade 1), fair (Grade 2), or poor (Grade 3).

Groups	n	Embryos G1		Embryos G2		Embryos G3	
	Total	(%)	n	(%)	n	(%)	n
CAPcontrol x FERTcontrol	15	33.3 ± 12.17	5	53.3 ± 12.88	8	13.3 ± 8.78	2
CAPcontrol x FERT1	27	40.7 ± 9.46	11	37.0 ± 9.29	10	22.2 ± 8.00	6
CAPcontrol x FERT10	23	43.5 ± 10.34	10	34.8 ± 9.93	8	21.7 ± 8.60	5
CAP1 x FERTcontrol	25	28.0 ± 8.98	7	24.0 ± 8.54	6	48.0 ± 9.99	12
CAP1 x FERT1	27	25.9 ± 8.43	7	37.0 ± 9.29	10	37.0 ± 9.29	10
CAP10 x FERTcontrol	25	32.0 ± 9.33	8	32.0 ± 9.33	8	36.0 ± 9.60	9
CAP10 x FERT10	19	36.8 ± 11.07	7	47.4 ± 11.45	9	15.8 ± 8.37	3

*The data is presented as the mean value ± standard error of the mean. CAPcontrol: capacitation medium without supplementation; CAP1: capacitation medium supplemented with 1 μM of UA; CAP10: capacitation medium supplemented with 10 μM of UA. FERTcontrol: fertilization medium without supplementation; FERT1: fertilization medium supplemented with 1 μM of UA; FERT10: fertilization medium supplemented with 10 μM of UA.

4. Discussion

The impact of UA on bovine spermatozoa capacitation and fertilization processes, and embryonic development was demonstrated for the first time in the present study. This antioxidant, at a concentration of 1 μM improved sperm linearity, straightness, and wobble and therefore the sperm movement quality. Moreover, a positive effect of UA supplementation seems to be reflected on the number of produced embryos. Although the technology for *in vitro* embryo production still requires considerable refinement, the production of higher number of good quality embryos can allow higher gestation rates and therefore, a higher number of neonates [31]–[33]. These results are of primordial importance for ART success [34].

Despite more than 60 years of optimization of semen cryopreservation protocols, the freezing process remains highly damaging for spermatozoa. In bovine artificial insemination centers, 40 – 50 % of sperm cells do not support cryopreservation, which generates high economic loses [32], [35]. Moreover, differences in the male reproductive ability for field reproduction compared to artificial insemination or embryo production, as in the present study, were often reported [24], [36]. It is very desirable to have a positive effect of UA supplementation on the gametes collected from different animals, especially of those with greater limitations in terms of fertility.

Several studies have already shown that UA regulates mitochondrial function, reduces ROS, prevents cellular damage, and various pathologies [2]–[4], [6], [37]. In the processes of *in vitro* fertilization, sperm cells, which are specialized cells with high energy consumption, are subjected to excessive ROS production during semen collection, cryopreservation and laboratory procedures [14], [15], [18], [38]–[40]. Oxidative stress is not only relevant in sperm but also in oocytes and is a major contributing factor to low oocyte maturation efficiency and deterioration of their quality, resulting in decreased fertilization and embryo production [41]. Therefore, UA by improving mitochondrial health and respiration might contribute to improve ART results. However, the biological effects of UA on gametes and embryos remain poorly characterized demanding further studies.

In the context of *in vitro* fertilization (IVF) systems, various parameters are commonly assessed to evaluate sperm quality, such as morphology, concentration, and motility [27]. However, more refined methodologies can be used to improve this evaluation, such as ATP and ROS production, OCR, and MMP. The primary function of mitochondria is to carry out the oxidative phosphorylation (OXPHOS), which results in the production of ATP, a vital metabolic energy source required for flagellar beating and sperm motility [42]. In the present study, UA supplementation at the highest concentration induced an increase of ATP production during the capacitation process. However, this dose (CAP10) has also reduced total motility with a decrease of rapid spermatozoa and increase of the static ones. According to Aitken, in 2017 [14] the loss of motility was one of the first and most-powerful impacts of oxidative stress on sperm. Conversely, a reduction of ROS levels was identified when spermatozoa were supplemented with the highest UA dose during capacitation. Recently, Santos and col. [20] have shown that the mitochondrial-targeted antioxidant, AntiOxBEN2, supplemented to the capacitation and fertilization media positively influenced both bovine sperm capacitation and fertilization processes, inducing a substantial reduction in ROS production, thereby improving early embryonic development. In accordance, Tripathi and col. [43], showed that the antioxidant supplementation reduced oxidative stress by decreasing ROS levels, thus improving embryo quantity and quality.

According to the results obtained, there was a tendency towards an increase of the OCR of capacitated sperm with 1 μM and 10 μM UA supplementation. As referred, OCR is a crucial indicator of mitochondrial activity, specifically of the electron transport rate. It serves as a representative measure of OXPHOS, the process by which spermatozoa utilized oxygen. In the case of bovine sperm, OXPHOS predominantly serves as their main energy-generating pathway [28], [29]. Accordingly, it was shown an increase of ATP levels of capacitated spermatozoa concomitant with a reduction of ROS levels at the highest concentration of UA. Thus, UA might have increased the mitochondrial function presenting some nuances in a dose-dependent manner. This result is relevant because excessive ROS can lead to oxidative stress and negatively affect sperm function during fertilization. ROS can be originated from both endogenous sources, such as sperm metabolism, and exogenous sources, like ART. It is important to maintain an appropriate balance of ROS to support sperm health and functionality [11], [14], [39], [44].

Presented results demonstrate, for the first time, the beneficial effect of UA on bovine sperm capacitation and *in vitro* fertilization. Although no significant differences were found in embryo quality rates, supplementation of CAP and/or FERT with UA increased the number of produced embryos compared to the untreated control group. The positive effect of UA is noteworthy. These results were obtained using semen of two bulls with distinct characteristics and demonstrates that UA supplementation provides a benefit in increasing *in vitro* embryo production. It would be interesting to conduct a more extensive approach, for example, by increasing the sample size of bulls with different reproductive characteristics to assess whether the results with UA were similar. Additionally, it could also be tested an intermediate concentration of UA, as the concentration of 10 μM showed better outcomes in reducing ROS and increasing ATP levels, while the concentration of 1 μM has induced better morphological and kinematic parameters, particularly in sperm movement quality.

The importance of ART and the expansion of reproductive biotechnologies in the context of animal production and reproductive efficiency has a significant socio-economic impact worldwide. The selection of breeds for fertility is highly significant, as they can contribute with valuable genetic material to their offspring, allowing for a greater selection differential compared to females [45]. Moreover, the use of cryopreserved semen contributes to maintain the genetic diversity within a population while mitigating the effects of inbreeding [46]. Therefore, the extensive implementation of antioxidant therapies, employed for the prevention, treatment, and enhancement of reproductive efficiency, holds unparalleled significance.

5. Conclusion

Obtained results showed that supplementation of UA at a concentration of 1 μM improved sperm movement quality, while the highest concentration decreased total motility and spz speed. A positive trend in increasing the oxygen consumption rate of capacitated sperm with both 1 μM and 10 μM UA supplementation was also identified. Additionally, 10 μM UA supplementation increased ATP and reduced ROS levels in capacitated sperm, indicating an enhanced mitochondrial function and reduced oxidative stress. Although no significant differences were observed in embryo quality rates, UA supplementation almost doubled the number of produced embryos. These results further support previous findings indicating the potential therapeutic value of UA in addressing reproductive sub/infertility problems and improving ART results making it a highly relevant and timely topic of interest that demand further research.

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