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The apoptotic inhibitor z-DEVD-fmk improves the viability and maturation rates of Iberian red deer oocytes while reducing apoptotic markers

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ABSTRACT

The main drawback in developing in vitro embryo production (IVP) systems in wild species, such as the Iberian red deer, is that access to these animals is usually restricted, and long distances from the collection site to the laboratory are usually inevitable. Prolonged ovary storage is known to negatively influence the quality and developmental competence of the oocytes used for IVP. To overcome this issue, we evaluated the effect of adding a caspase-3 inhibitor, z-DEVD-fmk, to the in vitro maturation media to improve the quality and developmental potential of Iberian red deer oocytes. Oocytes were in vitro matured with and without z-DEVD-fmk, and the following parameters were analyzed: viability, early apoptosis, caspase-3 activity, DNA fragmentation, and relative abundance of mRNA transcript related to apoptosis. Moreover, oocyte maturation and blastocyst rates were also assessed. The results showed that z-DEVD-fmk decreased early apoptosis (inhibitor = 44.44 ± 3.6% vs. control = 60 ± 2.79%), DNA fragmentation (inhibitor = 57.83 ± 1.91% vs. control = 74.62 ± 1.91%), caspase-3 activity (inhibitor = 41.88 ± 3.42% vs. control = 67.10 ± 3.42%) and the relative abundance of *TP53* and *ITM2B* transcripts, as well as increased the number of live (inhibitor = 41.48 ± 2.32% vs. control = 20 ± 1.8%) and in vitro-matured oocytes (inhibitor = 88.18 ± 1.99% vs. control = 74.01 ± 1.99%) rates. Nevertheless, the blastocyst production was not different between both experimental groups (inhibitor: 7.35 ± 2.30 vs. control: 13.77 ± 2.30). The supplementation of z-DEVD-fmk to the maturation medium improved the quality of Iberian red deer oocytes. Further research and alternative strategies are needed to evaluate if this inhibitor could still enhance the developmental potential of oocytes during prolonged ovarian transport.

1. Introduction

In the last decades, there has been a growing interest in using assisted reproductive technologies (ART) to conserve natural populations of Iberian red deer, a red deer subspecies distributed throughout the Iberian Peninsula [1–3]. There is a global shift in the popularity and interest of novel reproductive biotechnologies, such as in vitro embryo production (IVP), as it presents several advantages over more traditional methods. IVP's benefits as a reproductive tool are less hormone reliance, reduced cost of semen, a wider variety of donors, a shorter generational interval, and the preservation of valuable genetics.

Nevertheless, the application of IVP in wild species like the Iberian red deer has some limitations compared to other livestock species, further hampering the application of this technology to a broader extent. Access to these wild animals is usually restricted, and in many cases, they are found at great distances from the laboratories [4]. Previously, we showed that using sheep as a model for the transportation of ovaries beyond 7 h harmed the quality and developmental potential of oocytes and, therefore, the outcomes of IVP. Given that oocyte quality determines the developmental potential of embryos after fertilization [5], it is essential to preserve oocyte function before in vitro processing.

Immediately after collection, the occlusion of blood flow causes the ovaries not to receive oxygen and energy, leading to oxidative stress

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[6,7]. Moreover, oxygen deprivation results in hypoxia and a decrease in pH, as anaerobic metabolism is favored [8]. Ultimately, the hypoxic environment induces several cell death pathways in the follicle, such as apoptosis and necrosis [9,10]. In fact, during ovary transport, the number of oocytes showing DNA fragmentation, one of the biochemical hallmarks of apoptosis [11], is higher with increasing storage time [12]. Therefore, apoptosis is one of the processes that may be involved in the alteration of oocyte competence.

Caspases are a family of protease enzymes with crucial roles during apoptosis [13]. Depending on the structural characteristics and their role in apoptosis, caspases are generally subdivided into 3 major categories: inflammatory caspases (Group I: caspases-1, −4, −5, and −13), initiator caspases (Group II: caspases-2, −3 and −7), and effector or executioner caspases (Group III: caspases-6, −8, −9 and −10) [14]. Among them, caspase-3 directly degrades structural and functional proteins, causing DNA damage and cell death [15–17]. Recent studies showed that the inhibition of caspase-3 activity by z-DEVD-fmk in bovine oocytes and embryos blocked apoptosis induced by heat shock and tumor necrosis factor α [18,19]. Specifically, z-DEVD-fmk is a selective and irreversible inhibitor of caspase-3 that also shows potent inhibition of caspases − 6, − 7, − 8, and − 10. Indeed, this inhibitor can reduce neural injury after focal cerebral ischemia in rats [20,21] and improve cell survival in isolated human pancreatic islets [22] and platelets [23].

For this reason, we hypothesized that group II caspases, and more specifically caspase-3, are involved in the alteration of oocyte competence during prolonged ovary storage and that its inhibition may help mitigate the pro-apoptotic events and eventually enhance the efficacy of IVP protocols in Iberian red deer, where prolonged transport is inevitable.

In this regard, the present study aimed to evaluate the effects of the inhibitor z-DEVD-fmk on the quality and developmental competence of Iberian deer oocytes obtained from ovaries transported for prolonged times. Specifically, we decided to use the strategy of adding the inhibitor during in vitro maturation (IVM), based on previous studies performed by this group [12,24,25], in which apoptotic events due to prolonged ovarian transport were manifested in ovine oocytes only after IVM.

2. Materials and methods

The handling of the animals used during this experiment was in accordance with the Spanish policy for the proper care of animals, their exploitation, transport, experimental use, and sacrifice. (Law 32/2007). Unless otherwise specified, all reagents and chemicals were acquired from Merck Life Sciences (Madrid, Spain).

2.1. Experimental design

Two experimental groups were proposed (control and inhibitor). A control in which Iberian red deer oocytes from ovaries stored long times with melatonin were subjected to IVM without inhibitor, as well as an inhibitor group where the ovaries were stored in the same way and which the IVM medium was supplemented with the apoptosis inhibitor z-DEVD-fmk.

2.2. Oocyte retrieval and in vitro maturation

Ovaries from adult Iberian red deer (n = 140, approximately 35 in each of the 4 replicates) were collected post-mortem at a local licensed abattoir and transported in saline solution (8.9 g/L NaCl #S9625) containing penicillin (0.1 g/L #P3032), streptomycin (0.1 g/L #S6501), and melatonin (1 nM, Guinama S.L.U., La Pobla de Vallbona, Spain, #QC-00212845) at 25 °C during approximately 13 h. Upon arrival at the laboratory, immature cumulus-oocyte complexes (COCs) were collected from follicles with a surgical blade in 2 mL of recovery medium (TCM199 medium (#M4530) enriched with 2.38 mg/mL HEPES (#H4034), 2 μL/mL heparin (Hospira, Lake Forest, IL, USA; #654753.3) and 4 μL/mL gentamicin (#G1272)). Only COCs with either clear or moderately granular ooplasm, having at least three layers of packed cumulus cells (n = 350) were selected and transferred to the selection medium (TCM199 medium supplemented with 2.38 mg/mL HEPES and 4 μL/mL gentamicin). Subsequently, COCs were washed in TCM199 and 4 μL/mL gentamicin and homogenously distributed in 4-well dishes in two different groups with 500 μL of IVM media (TCM199, 4 μL/mL gentamicin, 100 μM of cysteamine (#M6500), 10 μg/mL of follicle-stimulating hormone (FSH), and 10% fetal calf serum (#F0804) with the following characteristics: control (IVM medium without inhibitor) and inhibitor (IVM medium supplemented with 200 nM z-DEVD-fmk (Santa Cruz Biotechnology, Inc. Dallas, USA, #sc-311558)). The COCs were incubated for 22 h at 38.5 ºC, 5% CO_2, and maximal humidity.

2.3. Nuclear maturation determination

Following IVM, a total of 214 oocytes (4 replicates) were washed in PBS-PVA (#P4417 and #P8136), removed from cumulus cells by gentle pipetting, and placed on a glass slide in a 1 μL drop of Slowfade™ (Invitrogen©, Thermo Fisher Scientific, Barcelona, Spain; #S36963) and 5 μg/mL Hoechst 33342 (#14533) under a coverslip. After 10 min at room temperature, chromatin patterns were assessed using fluorescence microscopy (Eclipse 80i, Nikon Instruments Europe, Amsterdam, The Netherlands). Oocytes exhibiting a germinal vesicle (GV) chromatin pattern were regarded as immature, and those showing a polar body and a metaphase plate were categorized as mature metaphase II (MII) oocytes.

2.4. Oocyte viability, early apoptosis, and necrosis evaluation

Oocyte viability, early apoptosis, and necrosis were determined in 60 matured oocytes (3 replicates) by using the Apoptotic, Necrotic & Healthy Cells Qualification Kit (Biotium©, San Francisco, USA; #30018), according to the manufacturer's guidelines. Denuded oocytes were incubated in a 30 μL drop of FITC Annexin V, Ethidium Homodimer III (EthD-III) and Hoechst 33342 for 20 min at room temperature and in the dark. Subsequently, oocytes were washed thrice in 1X Binding Buffer (diluting 5X Annexin Binding Buffer 1/5 with PBS-PVA) and mounted in slides in a 1 μL drop of Slowfade™. Oocyte nuclei and plasma membrane were observed with an epifluorescence microscope (Eclipse 80i, Nikon Instruments Europe, Amsterdam, The Netherlands) and classified into the following categories: viable (Annexin V − / EthD-III −), early apoptotic (Annexin V + / EthD-III −), and dead (Annexin V − / EthD-III + and Annexin V + / EthD-III +).

2.5. Analysis of caspase-3 activity

Caspase-3 activity was determined in 72 matured oocytes (4 replicates) using NucView® 488 Caspase-3 Assay Kit for live cells (Biotium©, San Francisco, USA; #30029-T). The NucView® 488 Caspase-3 substrate presents a binding site for caspase-3 and, upon cleavage, releases a high-affinity DNA dye. Following the manufacturer's protocol, denuded oocytes were incubated for 30 min in the dark and at room temperature in 30 μL drop of 5 μM NucView® 488 Caspase-3 substrate (diluted in PBS-PVA from a 200 μM substrate stock solution). Subsequently, oocytes were washed thrice in PBS-PVA and mounted on slides in a 1 μL drop of Slowfade™ with 5 μg/mL of Hoechst 33342. Fluorescence was detected on a Nikon A1 confocal microscope (Nikon Instruments Europe, Amsterdam, The Netherlands) using 408 and 488 nm lasers for Hoechst and green fluorescence, respectively. Fluorescent images were recorded. Mature oocytes were classified as caspase-3-positive if the nuclear DNA was stained with a bright green fluorescence.

2.6. Evaluation of DNA fragmentation

The TUNEL (Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) method was used to detect DNA fragmentation in matured oocytes (4 replicates) following the manufacturer's instructions. Seventy-two oocytes were fixed in 4% paraformaldehyde and then subjected to permeabilization in 0.5% Triton X-100 at room temperature for 1 h. Immediately, the oocytes were placed in 30 μL drop of TUNEL reagent containing fluorescein isothiocyanate conjugated with deoxyuridine 5-triphosphate (dUTP) incubated for 1 h at 37 ˚C. For the controls, we used previously fixed sheep oocytes to avoid losing Iberian red deer oocytes, which were limited. The positive control was preincubated with DNase I (0.2 U/μL; Thermo Fisher Scientific, Barcelona, Spain; #EN0521) for 1 h at 37 ˚C, whereas the negative control was incubated in the absence of the deoxynucleotidyl transferase enzyme. Images of the corresponding controls are depicted in Additional file 1. Afterward, oocytes were washed three times in PBS-PVA and placed on slides in a 1 μL drop of Slowfade™ with 5 μg/mL of Hoechst 33342. Samples were assessed on a Nikon A1 confocal microscope (Nikon Instruments Europe, Amsterdam, The Netherlands) using 408 and 488 nm lasers for Hoechst and green fluorescence, respectively. Oocytes showing DNA strand breaks were classified as TUNEL-positive.

2.7. Analysis of mRNA transcripts

The relative quantification of the mRNA transcripts of interest was performed by quantitative real-time PCR, according to the protocol described by Sánchez-Ajofrín et al., with slight modifications [25]. Briefly, RNA was extracted from 60 red deer oocytes using the Dynabeads® kit (Invitrogen, Waltham, CA, USA; #61012) in groups of approximately 10 oocytes (3 replicates). Subsequently, the Fermentas™ First-Strand cDNA Synthesis Kit (Thermo Scientific, Barcelona, Spain; #K1612) was used to reverse transcribe the mRNA samples and obtain the cDNA. The qPCR was performed on a LightCycler 480 II instrument (Roche, Barcelona, Spain). In each sample, 10 μL of NZYSpeedy qPCR Green Master Mix (Nzytech, Lisbon, Portugal; #MB22402), 400 nM of each of the forward and reverse primers, 2 μL of cDNA template, and 6.4 μL of nuclease-free water were added, reaching a final volume of 20 μL. PCR amplification consisted of the first step at 95 ˚C for 2 min, followed by 40 cycles of 95 ˚C for 5 s and 60 ˚C for 20 s. All samples were analyzed in duplicate, and reactions without any cDNA template (2 μL nuclease-free water) were used as negative controls. Right after, a melting curve analysis was carried out, and cycle threshold (Ct) values were registered. Comparative Ct and $2^{-\Delta\Delta CT}$ methods [26,27] were used to calculate the relative abundances of target transcripts: bcl2 associted X protein (*BAX*), b-cell lymphoma 2 protein (*BCL2*), caspase 3 (*CASP3*), SHC adaptor protein 1 (*SHC1*), tumor protein p53 (*TP53*), internal membrane protein 2b (ITM2B). The endogenous control (Peptidylprolyl Isomerase A (*PPIA*)) was used to normalize the quantification of candidate genes. Information on the qPCR primers is given in Additional file 2.

2.8. Cumulus cell's analysis

Cumulus cells were obtained from matured COCs of 4 replicates and examined by flow cytometry with the FlowSight® Imaging cytometer (Amnis, Merck-Millipore, Germany), as previously described [12]. To analyze viability, apoptosis, and necrosis, the samples were dyed with 10 μM of YO-PRO-1 (Thermo Fisher Scientific, Barcelona, Spain; #Y3603), which identifies apoptotic cells, and 0.5 μM of propidium iodide (PI) a dye for dead cells. To determine mitochondrial activity, cells were incubated with 200 mM of MitoTracker™ Deep Red (Thermo Fisher Scientific, Barcelona, Spain; #M22426) for 20 min at 38.5 ˚C in the dark and then counterstained with 10 μM of YO-PRO-1 and 0.5 μM of PI. Also, for the quantification of GSH and ROS intracellular levels in viable cells, samples were incubated with 10 μM of Cell Tracker™ Blue (Thermo Fisher Scientific, Barcelona, Spain) and 10 μM of CM-H_2DCFDA (Thermo Fisher Scientific, Barcelona, Spain) for 30 min at 38.5 ˚C, and then stained with 0.5 μM PI. The proportion of viable cells was classified as YO-PRO-1-/IP-, while apoptotic cells were identified as YO-PRO-1 + /IP-. Dead cells were IP + . Viable cells with active mitochondria were represented by the percentage of MitoTracker + /YO-PRO-1–. Finally, oxidative status was assessed only in viable cells by GSH and ROS production. A compensation matrix was applied prior to each experiment, and 1000 events per sample were recorded. In addition, IDEAS® software was utilized to analyze the raw data. A depicted image of cumulus cell populations in the IDEAS® software is shown in Additional file 3.

2.9. In vitro fertilization (IVF)

After IVM, a total of 112 COCs were distributed in 4-well dishes containing 500 μL of synthetic oviductal fluid (SOF [28]), enriched with 10% estrous sheep serum (ESS), and inseminated by IVF. For this purpose, oocytes were fertilized in vitro using frozen semen of one male deer supplied by the Medianilla S.L. company which is officially authorized for collecting and storing Iberian red deer semen. After thawing, spermatozoa were selected using a Percoll© density gradient (#P1644) (45%/90%) and capacitated for 15 min at 38.5 ˚C, and 5% CO_2 in SOF supplemented with 10% ESS. Finally, spermatozoa (10^6/mL) and oocytes were co-incubated for 18 h at 38.5 ˚C in 5% CO_2 and maximal humidity.

2.10. In vitro embryo culture

Following 18 h post-insemination (hpi), the presumptive zygotes were washed by repeated pipetting to eliminate spermatozoa and transferred to 25 μL drops (approximately one embryo per μL) of culture medium (SOF enriched with 3 mg/mL bovine serum albumin (#A9647)), coated with mineral oil. Presumptive zygotes were cultured up to day 8 post insemination (dpi) at 38.5 ˚C in a humidified atmosphere and 5% CO_2, 5% O_2, and 90% N_2 in the air. Cleavage rate was evaluated at 48 hpi, and blastocyst rate at 6,7 and 8 dpi. All expanded blastocysts from both treatments were fixed in 4% (v/v) paraformaldehyde and stored for TUNEL and cell number analysis.

2.11. Determination of blastocyst quality

To assess the blastocyst quality, total cell number and DNA fragmentation were determined in each embryo by combining TUNEL and Hoechst 33342 staining. Thus, fixed embryos of both treatments (3 replicates) were permeabilizated in 0.5% Triton X-100 at room temperature for 1 h, placed in 30 μL drop of TUNEL reagent, and incubated for 1 h at 37 ˚C. Subsequently, blastocysts were washed three times in PBS-PVA and placed on slides in a 1 μL drop of Slowfade™ with 5 μg/mL of Hoechst 33342. Negative and positive control samples were also included in the assay, in which we used expanded and fixed sheep blastocysts to avoid losing deer embryos. Positive controls were blastocysts exposed to DNase I (1 U/mL) for 15 min at room temperature, and negative controls were blastocysts not exposed to the kit's terminal TdT enzyme. Images of the corresponding controls are depicted in Additional file 4. Finally, blastocysts of both experimental groups were visualized with a Nikon A1 confocal microscope (Nikon Instruments Europe, Amsterdam, The Netherlands) using 408 and 488 nm lasers for total and apoptotic cell numbers, respectively (Fig. 1).

2.12. Statistical Analysis

Statistical analyses were performed using the IBM® SPSS® 24.0 (IBM Corp.; Armonk, NY, USA) software. Data were first tested for normal distribution (Kolmogorov–Smirnov, and Shapiro–Wilk tests). Then,

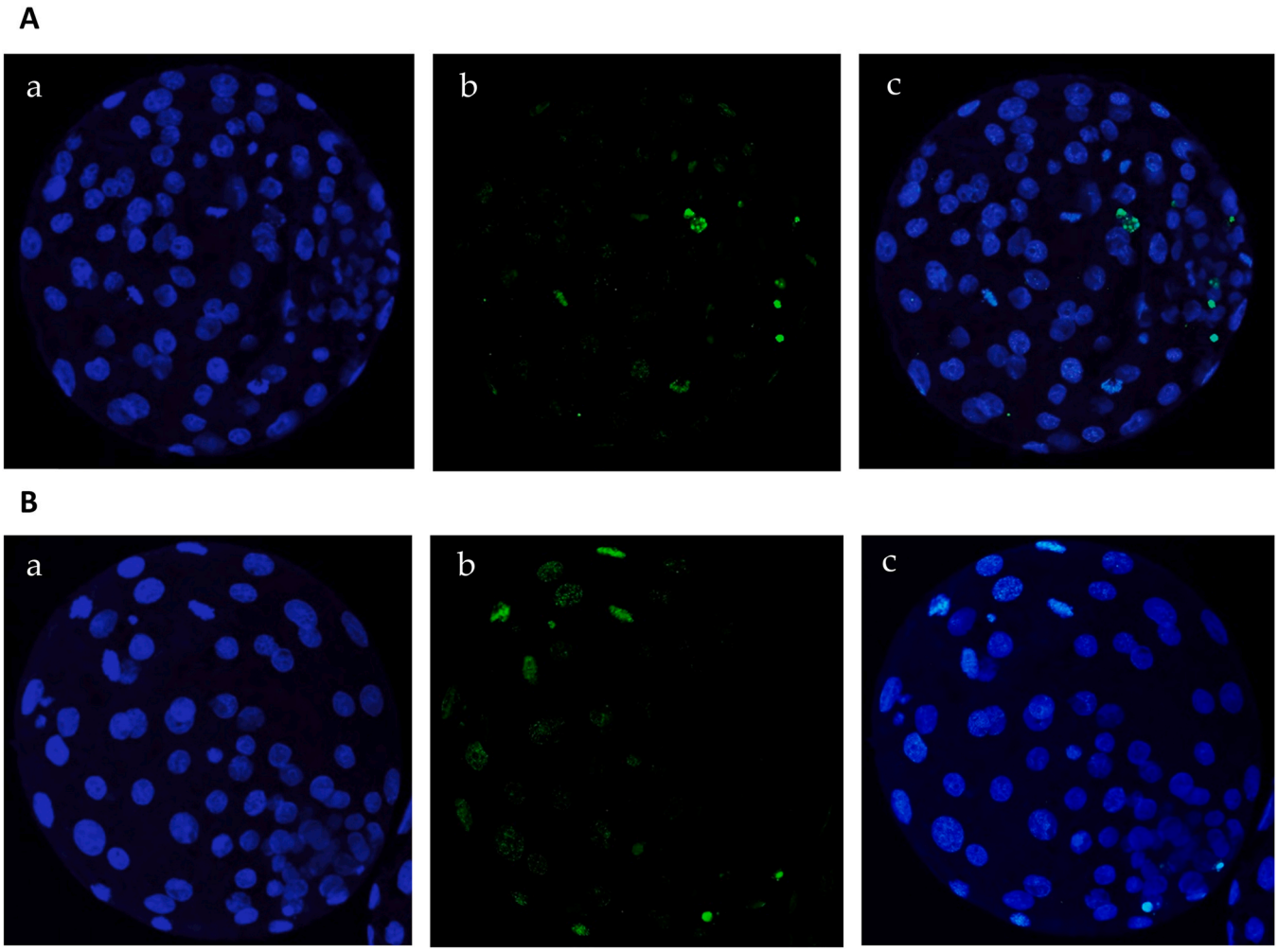

Fig. 1. A. Images representing a TUNEL-positive deer expanded blastocyst from non-inhibitor group (control) stained with (a) Hoechst 33342 and (b) fluorescein, and a (c) merged image. **B.** Images depicting a TUNEL-positive deer expanded blastocyst from Inhibitor group stained with (a) Hoechst 33342 and (b) fluorescein, and a (c) merged image.

DNA fragmentation, caspase-3 activity, oocyte maturation, and cumulus cell's parameters (4 replicates), oocyte viability, early apoptosis, necrosis, embryo development, and oocyte mRNA transcript abundance (3 replicates) were analyzed by factorial ANOVA followed by the Bonferroni post hoc test. Differences with probabilities of $p \leq 0.05$ were considered significant, and results are presented as corrected mean ± standard error of the mean (SEM).

3. Results

3.1. Determination of viability, early apoptosis, and necrosis in Iberian red deer oocytes after incubation with z-DEVD-fmk during IVM

As shown in Fig. 2, the percentage of viable oocytes was significantly increased ($p \leq 0.05$) in the inhibitor group (44.44 ± 2.32%) compared to the control (20 ± 1.8%). The percentage of dead oocytes was similar amongst the control and the inhibitor groups (14.07 ± 5.59% and 20 ± 4.33%, respectively; p-valor ≥ 0.05). However, the apoptotic oocytes were significantly decreased in the inhibitor group (41.48 ± 3.6%) compared to the control (60 ± 2.79%).

3.2. Assessment of Caspase-3 activity following the use of z-DEVD-fmk during oocyte IVM

The caspase-3 activity in oocytes incubated with inhibitor z-DEVD-fmk during IVM was also significantly reduced when compared to control oocytes (41.88% ± 3.42% and 67.10% ± 3.42, respectively, Fig. 3).

3.3. Evaluation of DNA fragmentation in Iberian red deer oocytes after using z-DEVD-fmk during IVM

DNA fragmentation, as measured by the TUNEL assay, had lower levels ($p \leq 0.05$) in oocytes treated with the z-DEVD-fmk inhibitor during IVM compared to those treated under control conditions (57.83% ± 1.91% and 74.72% ± 1.91, respectively, Fig. 4).

3.4. Assessment of apoptosis-related transcripts

We measured the relative abundance of mRNA transcripts of apoptosis-related genes in the oocytes. As shown in Fig. 5, there were no differences ($p \geq 0.05$) detected for *BAX*, *BCL2*, *CASP3*, *SHC1*, but the abundances of *TP53* and *ITM2B* were significantly lower ($p \leq 0.05$) in oocytes treated with z-DEVD-fmk during IVM.

3.5. Effects of apoptosis inhibitor z-DEVD-fmk on cumulus cells' quality parameters from in vitro matured Iberian red deer oocytes

To study the effect of the inhibitor on cumulus cells, several quality parameters were evaluated by flow cytometry. Cumulus cells obtained from COCs treated with the inhibitor showed similar values ($p \geq 0.05$) to those of the control treatment in the percentages of live/dead cells,

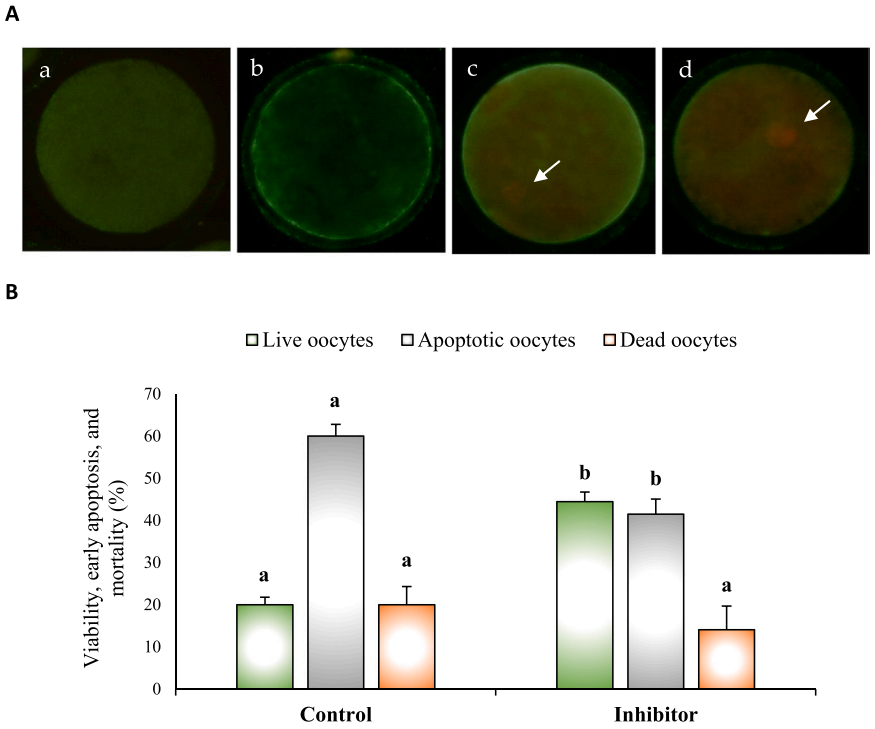

Fig. 2. A. Images depicting oocyte viability status. (a) Viable oocyte: Annexin V− / EthD-III − , (b) early apoptotic oocyte: Annexin V+ / EthD-III− , and (c) dead oocyte: Annexin V+ / EthD-III + (d) dead oocyte: Annexin V− / EthD-III+ . Arrows indicated the nucleus. **B.** Effect of z-DEVD-fmk during IVM on oocyte viability. Percentages of viable, apoptotic, and dead oocytes. Results are expressed as mean ± SEM. The same letters indicate no significant differences ($p \geq 0.05$) whereas different letters indicate significant differences ($p \leq 0.05$).

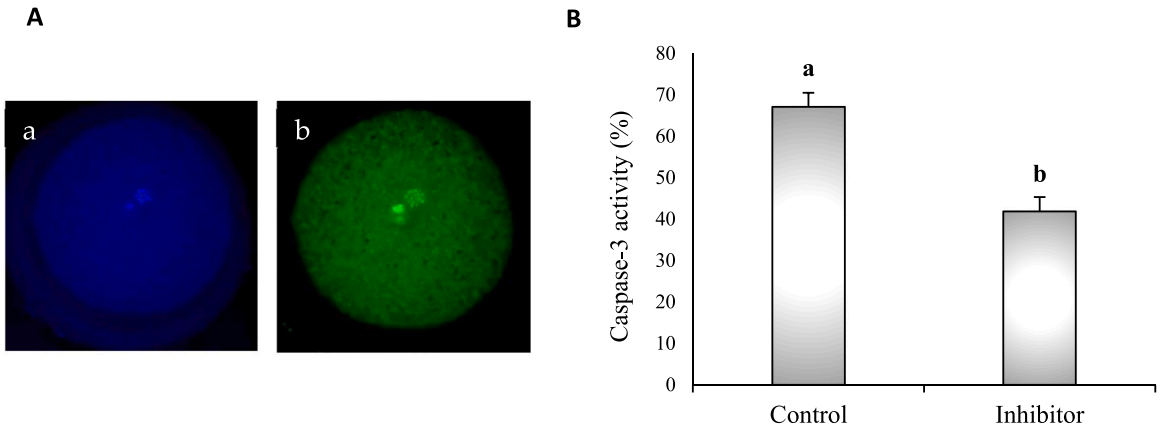

Fig. 3. A. Images depicting Caspase-3 staining. Caspase 3-positive MII oocyte stained with (a) Hoechst 33342 and (b) high- affinity DNA green dye. **B.** Effect of z-DEVD-fmk during IVM in Caspase-3 activity (%). Results are expressed as mean ± SEM. Different letters indicate significant differences ($p \leq 0.05$).

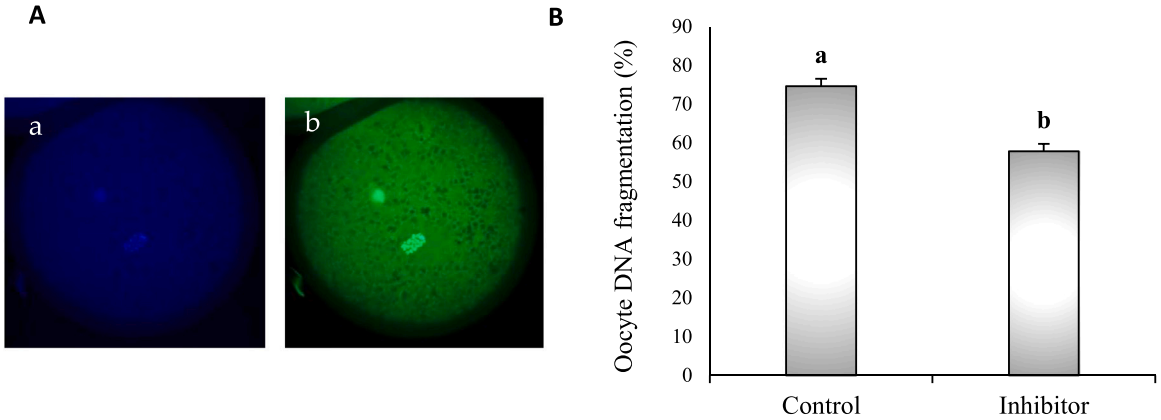

Fig. 4. A. Images representing a TUNEL-positive MII oocyte stained with (a) Hoechst 33342 and (b) fluorescein. **B.** Effect of z-DEVD-fmk on the DNA fragmentation in matured Iberian Red deer oocytes (%). Results are expressed as mean ± SEM. Different letters indicate significant differences ($p \leq 0.05$).

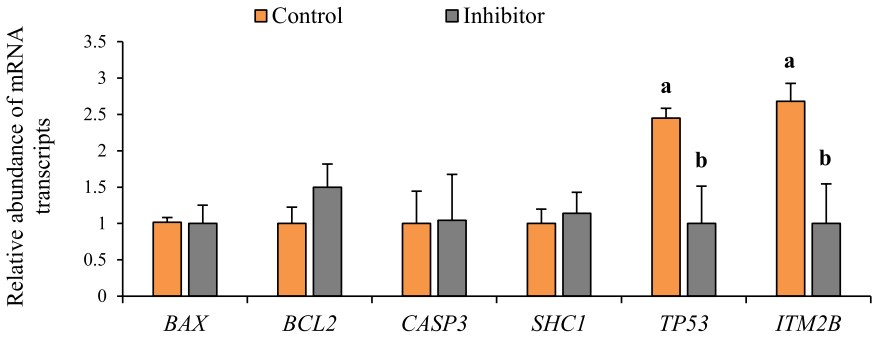

Fig. 5. Effect of z-DEVD-fmk on the relative expression of apoptosis-related genes. Results are expressed as mean ± SEM. Different letters indicate significant differences ($p \leq 0.05$).

Table 1
Effect of z-DEVD-fmk on the quality parameters of cumulus cells from IVM Iberian Red deer oocytes.

Treatment	Live (%)	Apoptotic (%)	Dead (%)	Active mitochondria (%)	ROS (fluorescence intensity)	GSH (fluorescence intensity)
Control	87.25 ± 1.68	0.53 ± 0.30	11.98 ± 1.42	53.20 ± 4.77	10655.01 ± 823.44	9931.03 ± 501.44
Inhibitor	84.32 ± 1.68	0.57 ± 0.30	15.02 ± 1.42	46.70 ± 4.77	12463.34 ± 823.44	11616.27 ± 501.44

Results are expressed as mean ± SEM. The results represent 4 replicates. There were not significant differences among treatments ($p \geq 0.05$).

apoptosis, active mitochondria, and GSH and ROS intracellular content (Table 1).

3.6. Effects of apoptosis inhibitor z-DEVD-fmk on the meiotic and developmental competence of Iberian red deer oocytes

The percentage of MII deer oocytes was higher ($p < 0.05$) in the inhibitor group compared to the control (88.18 ± 1.99% and 74.01 ± 1.99%, respectively, Fig. 6).

After IVM, red deer oocytes were inseminated and cultured to analyze if the inhibitor z-DEVD-fmk could improve their developmental competence. There were no differences ($p > 0.05$) between treatments in terms of cleavage, blastocyst, and blastocyst/cleavage rates (Table 2). Regarding blastocyst quality, the total cell number and TUNEL-positive blastomeres showed similar values between both treatments ($p > 0.05$; Table 2).

4. Discussion

To our knowledge, this research is the first to evaluate the use of the apoptotic inhibitor z-DEVD-fmk to ameliorate the detrimental effects of prolonged transport times on Iberian red deer oocytes. One of the main problems of IVP in Iberian red deer is that the oocytes are retrieved from ovaries collected from animals slaughtered in abattoirs which in many cases are far from the laboratory, leading to prolonged transportation [12,29]. During this process, the occlusion of blood flow reduces the supply of oxygen and energy to the follicles in the ovary. Considering that the ovarian tissue has higher metabolic rates than other tissues, there is a rapid accumulation of reactive oxygen species (ROS) in the follicular environment of the ischemic ovary [8,30,31]. Thus, the oocyte is subjected to an adverse oxidative environment that may compromise the cell's functions and, ultimately, lead to programmed cell death [9,16,32].

In this regard, improvements in the IVP protocol in Iberian red deer are needed to maintain the quality of the oocytes and, ultimately, their developmental competence during transport and subsequent in vitro processing. Thus, we previously addressed this issue by studying the effect of temperature and medium composition during the transportation of Iberian red deer [25,29] and sheep ovaries [24]. In particular, we found that adding melatonin to the ovary transport medium during prolonged storage improved cleavage and blastocyst rates [24,25]. Nevertheless, apoptosis-related markers (early apoptosis, DNA fragmentation, and mRNA transcripts) were also studied but showed no improvement when melatonin was added to the ovary storage medium in Iberian red deer experiments [25]. These results suggest that melatonin may not be able to prevent the activation of this type of cell death in the oocyte. For this reason, we considered a change in the strategy and decided to study the effect of the apoptosis inhibitor z-DEVD-fmk during IVM to examine if the quality of Iberian red deer oocytes after prolonged ovary storage could be improved and be reflected in a higher blastocyst yield after in vitro embryo production. The specific z-DEVD-fmk caspase-3 inhibitor has been previously added to the culture of different somatic

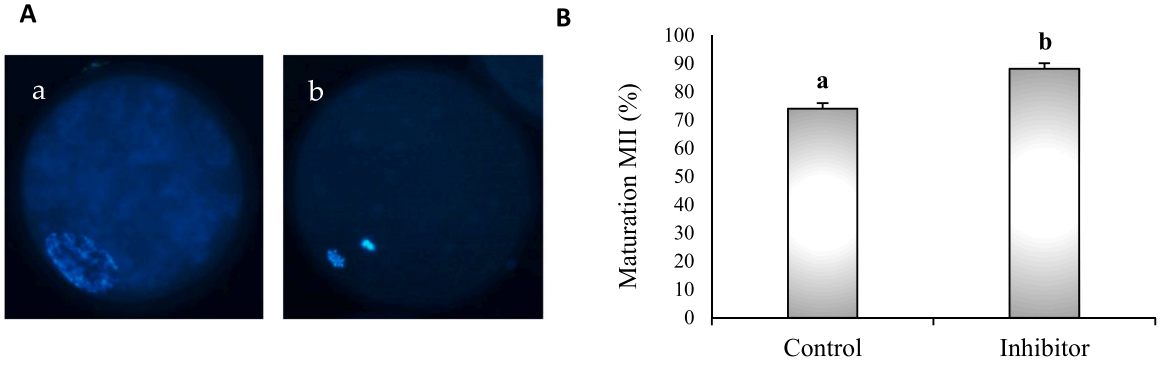

Fig. 6. A. Images representing (a) germinal vesicle (GV) stage oocytes and (b) MII oocytes. **B.** The effect of z-DEVD-fmk on the percentage of mature (MII) Iberian Red deer oocytes (%). Results are expressed as mean ± SEM. Different letters indicate significant differences ($p \leq 0.05$).

Table 2
Effect of z-DEVD-fmk on oocyte developmental competence and blastocyst quality in Iberian red deer.

Treatment	Total oocyte (n)	Cleaved Embryo at 48 hpi (%)	Total Expanded Blastocysts (%)	Expanded Blastocyst/ Cleaved (%)	Total cell number	TUNEL-positive Blastomeres (%)
Control	58	25.96 ± 4.36	13.77 ± 2.30	56.11 ± 23.57	123.17 ± 11.12	5.78 ± 1.25
Inhibitor	54	16.45 ± 4.36	7.35 ± 2.30	56.67 ± 23.57	134.88 ± 13.95	3.67 ± 1.56

Results are expressed as mean ± SEM. The results represent 4 replicates. There were not significant differences among treatments ($p \geq 0.05$).

cells [20–23], but has not been used extensively in the oocyte.

Our study shows that using z-DEVD-fmk during IVM of red deer oocytes effectively inhibits caspase-3 activity and decreases DNA fragmentation. These results agree with those observed by Hansen and Roth et al. (2004) in bovine heat-stressed oocytes, where the inhibitor z-DEVD-fmk also decreased caspase-3 activity and the percentage of DNA fragmentation [18]. Inactivation of the group II caspases, mainly the executioner caspase-3, leads to an induced interruption of apoptosis, possibly by halting the rapid and massive proteolytic degradation of the caspase cascade, in which key regulatory and structural proteins are shattered, and DNA is damaged [33,34]. Although apoptosis was initially regarded as an irreversible cell death process, several studies have reported that different cell types can reverse their apoptotic state in early stages [35–38] and in more advanced stages [34,39–41]. The natural ability of some cells to survive the onset of apoptosis is known as anastasis (Greek for 'rising life') and is characterized by mechanisms that allow cells to repair the damage once the apoptotic stress has been removed [40,42–45]. In this sense, the use of the z-DEVD-fmk inhibitor during IVM of Iberian red deer oocytes may induce anastasis, explaining, in part, the lower percentage of DNA fragmentation observed in this experiment. Indeed, studies performed on mouse fibroblast and lymphoma cultures, as well as HeLa cells reported that the inactivation of caspase-3 allows two of its substrates, the DNA repair enzyme poly (ADP) ribose polymerase (PARP) [46,47] and the inhibitor of caspase-activated DNase (ICAD) [48,49] are no longer cleaved. This may lead to DNA repair and decreased DNA fragmentation since PARP might still be active while caspase-activated DNase is inhibited.

In the present study, the use of the inhibitor also resulted in a lower number of oocytes in early apoptosis and a higher number of live oocytes. Phosphatidylserine (PS) resides in the inner leaflet of the plasma membrane under normal conditions, but it is exposed on the outer surface during early apoptosis as a signal that facilitates the cell's rapid removal by phagocytes [50,51]. Once PS is exposed to the cell surface, it can be attached to Annexin V, a family of phospholipid-binding proteins with unknown functions [52]. Although the mechanism of PS externalization is unclear, the lower percentage of Annexin V+ oocytes observed after the incubation with z-DEVD-fmk in our study can be directly related to reduced induction of apoptosis in the oocyte.

Additionally, the analysis of apoptosis-related genes showed a decrease in the relative abundance of *TP53* and *ITM2B* mRNA transcripts in the oocytes of the inhibitor group. As widely known, TP53 codifies for tumor protein p53, a key cell cycle regulator that can promote apoptosis in response to a variety of insults, including hypoxia, whose activation leads to the upregulation of several pro-apoptotic genes responsible for the apoptotic phenotype and ultimately cell demise [32,33,35]. ITM2B codifies for a BH3-only integral membrane protein identified as a p53-independent regulator of apoptosis [53]. Therefore, our results suggest that z-DEVD-fmk may suppress the p53-dependent apoptotic response and ITM2B-mediated apoptosis, and thus enabling the possible anastasis.

Furthermore, the incubation with inhibitor z-DEVD-fmk during IVM increased the maturation rates of Iberian red deer oocytes. The higher percentage of MII oocytes may be associated with the inhibition of apoptosis by z-DEVD-fmk, since a greater number of live oocytes were also observed after this treatment. However, these positive results did not lead to a significantly increase in cleaved embryo and blastocyst rates and may even appear to have a negative impact. Similar to our results, Colombo et. al, (2020) showed that while pan-caspase inhibitor z-VAD-fmk during vitrification of cat oocytes reduced DNA fragmentation and caspase activity, and increased maturation rates after IVM, embryo yield was not improved [54]. Nuclear maturation encompasses the processes of reversing meiotic arrest at prophase I and driving the progression of meiosis to metaphase II. These changes are essential to confer the oocyte the capacity to undergo normal fertilization and sustain embryonic development [55]. In addition, cytoplasmic maturation is also necessary for the production of fertilizable oocytes [56]. In fact, to produce embryos in vitro with increased developmental potential, the culture system needs to adequately support the oocyte's nuclear maturation and cytoplasmic competency [57]. In our study, it is possible that the anastasis induced by z-DEVD-fmk was not sufficient to cope with the damage produced in the oocyte by prolonged transport, making possible the survival of oocytes with alterations in their structure that are not suitable for producing an embryo. Therefore, considering the present results we cannot affirm that the inhibitor has negative effects, but rather that the inhibitor most likely does not have an effect on other events that compromise oocyte competence. Indeed, one of the major problems that can result from anastasis, especially in cells with more advanced apoptosis, is the high probability of genetic abnormalities caused by unrepaired DNA damage [34,41,43,54], which, in the case of oocytes, could compromise their developmental competence or entail the risk of transmitting new mutations to the progeny [34,54]. In this way, the combination of possible failures in the genome of the rescued oocytes, together with a failed or incomplete cytoplasmic maturation, common in IVP procedures [57–59], in which the inhibitor most likely had no effect, could be the main cause of the lack of improvement in the blastocyst yield.

One strategy to improve the embryo production outcomes and exclude the possibility of a negative impact may be using z-DEVD-fmk in stages before or after IVM, i.e., during ovary transport and IVF-IVC. Firstly, adding z-DEVD-fmk to the ovary storage medium may prevent the damage to oocytes caused by premature apoptosis and reduce the likelihood of more serious injuries that could be more difficult to repair. Secondly, using z-DEVD-fmk during IVF and IVC may also enhance oocyte developmental competence. However, its application during IVC might be controversial since apoptosis is a natural process during embryo development to allow the selection of healthy blastomeres [60]. A study by Paula-Lopes and Hansen (2002) found that using inhibitor z-DEVD-fmk in the embryo culture medium of heat-stress bovine embryos was indeed detrimental [61]. On the contrary, Mullani et al. (2016) observed that using the inhibitor z-LEHD-fmk, a caspase-9 inhibitor, during buffalo embryo IVC enhanced the number of pre-implantation embryos [62]. In this sense, future studies will be necessary to evaluate the survival and developmental potential of Iberian red deer embryos and oocytes incubated with apoptosis inhibitors during the different stages of IVP and to see if, when transferred to recipient females, they eventually give rise to a healthy offspring.

5. Conclusions

In vitro maturation of Iberian red deer oocytes with the inhibitor z-DEVD-fmk decreased early apoptosis, DNA fragmentation, caspase-3 activity, and the relative abundance of *TP53* and *ITM2B* mRNA

transcripts, as well as increased the viability and maturation rates. However, it did not improve blastocyst yield or quality. To corroborate the possible positive effects of the inhibitor on Iberian red deer embryo production, it will be interesting to include z-DEVD-fmk during prolonged ovary transport and at later stages of the IVP protocol. Likewise, it is still important to continue studies related to oocyte damage during prolonged transport of Iberian red deer ovaries to elucidate the molecular mechanisms involved and find new targets that can improve oocyte developmental competence.

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CRediT authorship contribution statement

"D.A.M.-C.; J.A.L and I.S.–A. analyzed and interpreted the data, J.A.L.; O.G.-A., J.A.O.-M.; C.M.P., A.M.M.; M.R.F.-S. collected the samples, D.A.M.-C. and I.S.–A wrote the manuscript and J.J.G. and A.J.S reviewed it. All authors read and approved the final manuscript.".

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.therwi.2023.100021.

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