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Effect of caspase inhibitor Z-VAD-FMK on bovine sperm cryotolerance

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Abstract

The aim of this study was to evaluate the treatment of bovine semen with the pan-caspase inhibitor benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-FMK), before or after freezing on semen quality. After the initial assessment, sperm from 4 bulls were pooled (Experiment 1) and cryopreserved in BioXcell containing 0, 20 and 100 μM Z-VAD-FMK. After thawing semen viability, motility, membrane integrity, as well as DNA fragmentation and ΔΨm were evaluated. In Experiment 2, bovine frozen/thawed sperm were incubated for 1 hr with 0, 20 and 100 μM Z-VAD-FMK before assessing the semen quality. The treatment with Z -VAD-FMK before cryopreservation improved post-thawing sperm motility compared to the control group ($p < .05$), while no differences were recorded in sperm viability and membrane integrity among groups (on average 86.8 ± 1.5 and 69.1 ± 1.4, respectively). Interestingly, at the highest concentration, DNA fragmentation decreased ($p < .05$), while the percentage of spermatozoa with high ΔΨm increased ($p < .05$). The results of Experiment 2 showed that 1-hr treatment with Z-VAD-FMK did not affect sperm motility and viability (on average 63.4 ± 5.8 and 83.7.1 ± 1.2, respectively). However, Z-VAD-FMK improved sperm membrane integrity ($p < .05$) and at the highest concentration tested decreased the proportion of sperm showing DNA fragmentation ($p < .05$). No differences were recorded in the percentage of spermatozoa with high ΔΨm (on average 57.0 ± 11.4). In conclusion, the treatment with 100 μM of the caspase inhibitor Z-VAD-FMK before freezing increased bovine sperm mass motility and ΔΨm, while decreasing sperm DNA fragmentation. Treatment of semen after thawing with 100 μM Z-VAD-FMK improved sperm membrane integrity and reduced DNA fragmentation.

KEYWORDS

apoptosis, bovine semen, caspase inhibitor

1 | INTRODUCTION

Semen cryopreservation is a valid tool of livestock industry, which has greatly improved the diffusion of artificial insemination in cattle since the 1960s, allowing long-term storage and transport of germ plasm. Nevertheless, cryopreservation, although maintaining sperm viability at subzero temperatures, determines deleterious effects on cell structures resulting in reduced fertility (Pegg, 2015; Woods, Benson, Agca, & Critser, 2004). It is known that cryopreservation

decreases motility, membrane integrity and mitochondrial activity, induces capacitation-like changes, hence reducing viability and fertilizing ability (Longobardi et al., 2017; Parks & Graham, 1992). It follows that improvement of semen cryopreservation would favourably impact on the efficiency of reproductive technologies intervening on both the paternal and maternal lineages for accelerating genetic progress.

During the cryopreservation process, several mechanisms, including mechanical/osmotic injury and oxidative stress, alter the physical properties of cellular structures inducing activation of the apoptotic pathways and consequently cell death (Baust, Buskirk, & Baust, 2000; Paasch et al., 2004). It is known that sperm DNA integrity is essential for the accurate transmission of genetic information and, in healthy condition, ejaculates contain two populations of spermatozoa: a non-apoptotic fraction containing morphologically superior quality sperm, and an apoptotic fraction associated with increased abnormal sperm morphology (Anzar, He, Buhr, Kroetsch, & Pauls, 2002; Aziz, Said, Paasch, & Agarwal, 2007; Peña, Johannisson, Wallgren, & Rodríguez-Martínez, 2003).

Although apoptosis is a physiological event of programmed cell death, various stressors, including cryopreservation, may trigger an abnormal activation of the apoptotic pathway leading to cell degeneration (Martin, Sabido, Durand, & Levy, 2004). The complex phenomenon of apoptosis occurs through the three phases of induction, execution and degradation (Martin et al., 2004). Mitochondria are involved in the execution phase, during which the mitochondrial pores open, leading to decreased $\Delta\Psi m$ and activation of pro-apoptotic factors, such as proteases related to the caspase family in the cytoplasm (Ravagnan, Roumier, & Kroemer, 1997). The activation of caspases leads to the degradation phase of apoptosis, where changes at the cell surface and nucleus occur, like translocation of phosphatidylserine from the inner to the outer leaflet of plasma membrane, and DNA fragmentation (Bratton et al., 1997). It has been demonstrated that cryopreservation induces cell degeneration by activating the apoptotic pathway in different cells, including oocytes and embryos of domestic species (Baust et al., 2000; Men, Monson, Parrish, & Rutledge, 2003; Paasch et al., 2004). This pathway is triggered by the activation of caspases, a group of proteolytic enzymes, playing a key role in the execution of apoptosis (Zhivotovsky, Burgess, Vanags, & Orrenius, 1997).

Interestingly, the inhibition of caspase activity was reported to prevent apoptosis and improve cryotolerance of mammalian cells (Stroh et al., 2002; Yagi et al., 2001). Likewise, an increased cryotolerance of porcine and bovine embryos was also recorded by inhibiting apoptosis using a caspase inhibitor, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-FMK), during vitrification and subsequent culture (Men, Agca, Riley, & Critser, 2006; Pero et al., 2017). Z-VAD-FMK is a known pan-caspase inhibitor that binds to the catalytic sites of caspases 3, 8 and 9, preventing the activation of the initiators (caspase 9 and 8) and/or the effector (caspase 3) of the apoptotic cascade. The incubation with a caspase inhibitor prior vitrification improved survival, cleavage and embryo yields of porcine oocytes (Niu, Jianjun, Chen, Wu, Zhang, & Zhang, 2016). Contradicting results are reported on the effects of caspase inhibition on sperm cryotolerance in different species. Indeed, caspase inhibition was effective at improving sperm fertility parameters involved in the apoptosis pathway, suggesting a possible function to prevent apoptosis-like changes in post-thawed buffalo sperm (Dalal, Kumar, Honparkhe, & Brar, 2019; Dalal, Kumar, Honparkhe, & Singh., Singh, A.K., Brar, P.S., 2018). In contrast, treatment with Z-VAD-FMK failed to improve cryotolerance of equine and canine sperm (Peter, Colenbrander, & Gadella, 2005; Peter & Linde-Forsberg, 2003). To the best of our knowledge, the strategy to prevent apoptosis by inhibiting caspase activity has not yet been evaluated on bovine semen.

It was hypothesized that the inhibition of caspases by Z-VAD-FMK may prevent cryopreservation-induced aberrant apoptosis and hence improve cryotolerance of bovine sperm. Therefore, the aim of this study was to evaluate whether the treatment of bovine semen with the pan-caspase inhibitor Z-VAD-FMK before or after freezing improves post-thawing sperm viability, motility, membrane integrity and mitochondrial membrane potential and reduces DNA fragmentation.

2 | MATERIALS AND METHODS

Unless otherwise stated, reagents were purchased from Sigma-Aldrich—Merck. The RNA-free DNase and RNAse A were obtained from Roche Diagnostics Corporation, while the pan-caspase inhibitor Z-VAD-FMK from Promega Corporation.

2.1 | Experimental design

No ethical approval was obtained because this study did not involved laboratory animals and only involved non-invasive procedures. Twelve healthy Holstein Friesian (Bos Taurus) bulls (4–6 years age) maintained at an authorized National Semen Collection Center (Centro Tori Chiacchierini) under uniform management conditions, routinely used for semen collection twice per week, were selected for the trial. Semen was collected using an artificial vagina prewarmed to 42°C, and only the ejaculates with ≥70% motility were utilized. A total of 48 ejaculates (4/bull) were used for the trial, and in order to reduce individual variability, on each day of collection semen from 4 bulls was pooled together.

In Experiment 1, each pool was divided into 4 aliquots: one for analyses of fresh semen, while the other three aliquots were diluted at 37°C with an animal-free protein extender BioXcell (IMV-technologies, France), containing 0 (control group), 20 μM and 100 μM Z-VAD-FMK to a final concentration of 30×10^6 spermatozoa/ml. The diluted semen was packaged in 0.5 ml French straws and subjected to a combined cooling with equilibration period of 3 hr at 5°C. The straws were kept in automatic programmable biological cell freezer (IMV technology, France) until temperature of straws reached −145°C. Then, straws were plunged into liquid nitrogen (−196°C) for storage until analyses

(4–8 weeks). After thawing at 37°C for 40 s in a water bath, sperm viability, motility, membrane integrity and DNA fragmentation were evaluated. In addition, the mitochondrial membrane potential (ΔΨm) was assessed by JC-1 staining (5,5,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine iodide) by flow cytometry.

In Experiment 2, to evaluate the effect of Z-VAD-FMK after freezing, frozen semen (n = 36 ejaculates) was thawed, washed and sperm were incubated for 1 hr with 0, 20 and 100 μM Z-VAD-FMK before assessing the fertility parameters described for Experiment 1.

2.2 | Z-VAD-FMK preparation

The caspase inhibitor Z-VAD-FMK (G-7232) stock solutions (20 and 100 mM in dimethyl sulfoxide) were aliquoted and frozen until day of use. Then, to obtain the desired concentrations (20 and 100 μM) and to reduce to the minimum the amount of dimethyl sulfoxide (DMSO) during freezing/ thawing, the caspase inhibitor Z-VAD-FMK stock solutions were diluted 1:1,000 in the extender. Likewise, in the control group, DMSO (1:1,000) was added in the extender.

2.3 | Assessment of post-thawing sperm motility

Sperm motility on fresh and frozen/thawed semen was examined by phase-contrast microscopy (Nikon E200) at 40× magnification on a clean and dry glass slide overlaid with a coverslip and maintained on thermo-regulated stage at 37°C. Any drifting of the specimen was permitted to stop and the percentage of motile spermatozoa was subjectively determined to the nearest 5% by analysing four to five fields of view (Longobardi et al., 2017).

2.4 | Assessment of sperm viability by Trypan blue/ Giemsa technique

The viability was assessed by Trypan Blue/Giemsa technique as reported by Boccia, Di Palo, De Rosa, Attanasio, Mariotti, & Gasparrini. (2007). Briefly, on a clean slide, 5 μl of semen and 5 μl 0.27% Trypan blue were spread, fixed for 2 min and stained with 7.5% Giemsa overnight. Sperm cells were microscopically evaluated at 40× magnification (Nikon E200) and differentiated as live, that is only sperm displaying both head and tail viable (pink coloured) and as dead, that is those with either the head or the tail unviable (black-dark violet coloured). A total of 200 spermatozoa were analysed per slide.

2.5 | Assessment of sperm membrane integrity

Sperm membrane integrity was assessed by the HOS test, as described by Jeyendran, Van der Ven, Perez-Pelaez, Crabo, & Zaneveld. (1984). Fifty microliter of semen was mixed with 500 μl of a hypo-osmotic solution (0.73 g sodium citrate and 1.35 g fructose in 100 ml of distilled water, 150 mOsm) and incubated at 37°C for 45 min. A drop of diluted semen was placed on a clean slide and covered with a cover slip. A total of 200 spermatozoa were counted in different fields at 40X under phase-contrast microscope (Nikon E200) and the percentage of spermatozoa positive to HOS test (having coiled tails) was determined.

2.6 | Evaluation of DNA fragmentation by Tunel assay

Freshly ejaculated and cryopreserved spermatozoa after thawing (37°C/40 sec) were washed in Modified Sperm Tyrode's albumin lactate pyruvate medium (Sperm-Talp) according to Parrish, Susko-Parrish, Leibfried-Rutledge, Critser, Eyestone, & First. (1986). For washing, 0.5 ml of freshly ejaculated or frozen-thawed semen were diluted in 2 ml of Sperm-Talp, mixed gently and centrifuged twice at 300 g for 10 min. Supernatant was discarded, and sperm concentration was adjusted to 20×10^6 cells/ml in Sperm-Talp.

The amount of DNA fragmentation was determined by Tunel assay using a commercially available kit (In Situ Cell Death Detection Kit, fluorescein, Roche). For each sample, 100 μl of washed sperm was fixed with 4% (w/v) paraformaldehyde in phosphate-buffered saline (PBS) for 30 min at room temperature, washed two times in PBS with 0.1% polyvinylpyrrolidone (PVP) through centrifugation at 300 g for 10 min, smeared on glass slides and air-dried. Samples were permeabilized in 0.1% Triton X-100 in 0.1% sodium citrate for 10 min, washed in PBS-PVP two times and then incubated in TUNEL reaction mixture according to the manufacturer for 1 hr at 37°C in a dark and humidified atmosphere. For a positive control, slides were treated with RNase-free DNase I at room temperature for 10 min before incubation with the TUNEL reagent. For a negative control, slides were incubated with the TUNEL reagent in the absence of terminal deoxynucleotidyl transferase. At the end of incubation, slides were washed in PBS-PVP labelled with Hoechst 33342 1 mg/ml for 30 min at room temperature, washed again in PBS-PVP and mounted onto a glass microscope slide in a drop of glycerol and flattened with a coverslip. At least 200 spermatozoa were analysed in each sample by using a fluorescent microscope (Eclipse E-600; Nikon, Japan) under ultraviolet light with excitation DAPI (460 nm for blue fluorescence) and FITC (520 nm for green fluorescence) filters. Digital images of each spermatozoa were acquired using NIS-Elements-F software and a high-resolution colour digital camera (Digital Sight DS-Fi 1C; Nikon), and the numbers of total (blue) and TUNEL-positive (green) nuclei were recorded (Figure 1).

2.7 | Evaluation of mitochondrial membrane potential (ΔΨm)

The mitochondrial membrane potential was evaluated by JC-1 staining (Thermofisher Scientific, T3168), as described before

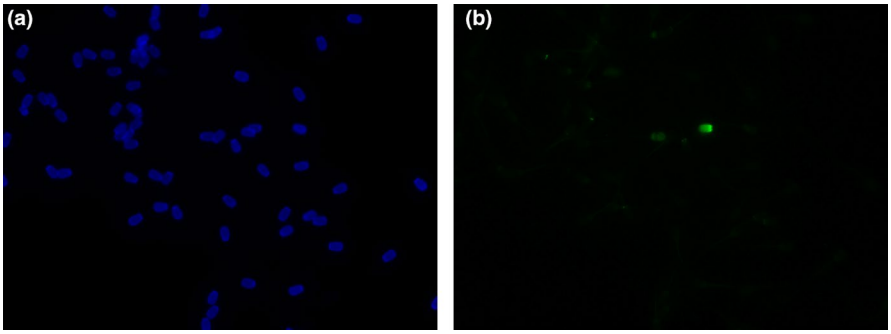

FIGURE 1 Representative images of Hoechst-stained (a) and TUNEL-positive (b) spermatozoa

TABLE 1 Percentage of sperm motility, viability, membrane integrity (HOS-positive cells) and DNA fragmentation (Tunel-positive cells) in fresh and frozen semen (4 ejaculates per 12 bulls)

	Fresh Mean ± *SE*	Frozen Mean ± *SE*
Motility (%)	73.3 ± 1.1^{a}	70.0 ± 1.05^{b}
Viability (%)	89.3 ± 1.9	86.3 ± 1.3
Membrane integrity (% HOS+)	73.8 ± 1.6^{a}	67.1 ± 1. 5^{b}
DNA fragmentation (% Tunel+)	1.1 ± 0.6^{A}	15.4 ± 2.8^{B}

Note: Values within rows with different superscript uppercase letters are significantly different; $p < .01$.

Values within rows with different superscript lowercase letters are significantly different; $p < .05$.

TABLE 2 Sperm motility, viability, membrane integrity (% of HOS-positive cells), ΔΨm and DNA fragmentation (% of Tunel-positive cells) in groups treated with 0, 20 μM and 100 μM with the caspase inhibitor Z-VAD before freezing (4 ejaculates per 12 bulls)

	Groups		
	0 (control) Mean ± *SE*	20 μM Z-VAD Mean ± *SE*	100 μM Z-VAD Mean ± *SE*
Motility (%)	70.0 ± 1.3^{Aa}	80.0 ± 1.3^{B}	75.0 ± 1.3^{b}
Viability (%)	87.1 ± 1.0	84.3 ± 1.0	85.0 ± 2.4
HOS+ (%)	67.1 ± 1.2	71.5 ± 1.7	68.8 ± 1.1
ΔΨm (%)	62.5 ± 2.6^{ab}	51.3 ± 4.7^{a}	71.4 ± 5.8^{b}
Tunel+ (%)	16.9 ± 2.4^{a}	13.3 ± 1.6^{ab}	9.6 ± 2.0^{b}

Note: Values within rows with different superscript uppercase letters are significantly different; $p < .01$.

Values within rows with different superscript lowercase letters are significantly different; $p < .05$.

(Hendricks, & Hansen, 2009; Ortega-Ferrusola et al., 2008). The JC-1 has the unique ability to differentiate mitochondria with low and high ΔΨm. In the high ΔΨm mitochondria, JC-1 assembles multimeric aggregates that exhibit a red fluorescence (590 nm). If excited simultaneously from argon ion laser sources (488 nm), monomers and aggregates can be detected separately by flow cytometry in FL1 and FL2 channels. Spermatozoa were washed as previously described and 500 μl incubated for 15 min at 37°C in the dark with 0.5 μl of JC-1 (2 mM). At the end of incubation, samples were analysed using the FACSCaliburT flow cytometer (BD Biosciences). At least, 50,000 events per sample were analysed and three sperm subpopulations have been identified: spermatozoa with high ΔΨm (red fluorescence), spermatozoa with low ΔΨm (green fluorescence) and spermatozoa with heterogeneous mitochondria, with high and low ΔΨm (red and green fluorescence).

2.8 | Statistical analysis

Differences in sperm motility, viability, membrane integrity and DNA fragmentation between fresh and frozen semen were analysed by Student's *t* test. In Experiments 1 and 2, the differences among groups in motility, viability, membrane integrity, DNA fragmentation and mitochondrial membrane potential were analysed by ANOVA, with Tukey's test used for post hoc comparisons.

3 | RESULTS

The fertility parameters of fresh semen, such as motility, viability, membrane integrity and the DNA fragmentation, showed the good quality of the semen before cryopreservation (Table 1). Despite similar viability, freezing reduced ($p < .05$) sperm motility and membrane integrity (Table 1). Furthermore, the percentage of sperm showing DNA fragmentation dramatically increased ($p < .01$) in frozen semen as opposed to fresh semen (Table 1).

3.1 | Experiment 1. Treatment with Z-VAD-FMK prior freezing

As shown in Table 2, treatment with both concentrations of Z-VAD-FMK before cryopreservation improved post-thawing sperm motility compared to the control group ($p < .05$). In the treated groups, motility also varied in terms of pattern compared to the control group, as sperm moved with vigorous and sudden movements. The treatment with both concentrations of Z-VAD-FMK affected neither sperm viability nor membrane integrity that were high in all groups upon thawing (Table 2). However, at the highest concentration (100 μM) Z-VAD-FMK decreased ($p < .05$) the proportion of sperm exhibiting DNA

fragmentation (Table 2). Furthermore, the percentage of spermatozoa with high mitochondrial membrane potential increased in 100 μM Z-VAD-FMK treated group compared with 20 μM Z-VAD-FMK treated group ($p < .05$) and to the control group ($p = .08$), as shown in Table 2.

3.2 | Experiment 2. Treatment of frozen-thawed sperm with Z-VAD-FMK

As shown in Table 1, 1-hr treatment of frozen-thawed sperm with the caspase inhibitor Z-VAD-FMK did not affect sperm motility and viability. However, the inhibitor was effective at improving sperm membrane integrity both at 20 μM Z-VAD-FMK ($p < .05$) and at 100 μM Z-VAD-FMK ($p < .01$). At the highest concentration (100 μM), Z-VAD-FMK also decreased ($p < .05$) the proportion of sperm showing DNA fragmentation (Table 3). No differences were recorded in the percentage of spermatozoa with high mitochondrial membrane potential (Table 3).

4 | DISCUSSION

The rationale of this work was to verify whether the inhibition of caspase by Z-VAD-FMK FMK before freezing or after thawing would prevent cryopreservation-induced apoptosis, improving quality of bovine semen. The results of the study demonstrated that treating sperm prior freezing with 100 μM Z-VAD-FMK results in increased motility, decreased $\Delta\psi_m$ and reduced DNA fragmentation, without affecting viability and membrane integrity. On the other hand, the incubation of thawed sperm with the inhibitor improved sperm membrane integrity and decreased DNA fragmentation.

Cryopreservation and/or thawing has a negative impact on sperm viability, due to the formation of ice crystals that can damage the membrane and alter the cytoskeleton and the functions of cytoplasmic organelles (Aziz et al., 2004). Alterations in membrane permeability, mitochondrial damage and oxidative stress trigger a series of events leading to cellular death (Wyllie, Kerr, & Currie, 1980). Cell degeneration caused by the insults related to semen cryopreservation and thawing procedures primarily occurs through apoptosis (Martin et al., 2004). In the present study, cryopreservation significantly reduced mass motility and the percentage of intact-membrane spermatozoa, parameters known to affect the fertilizing ability of semen (Medeiros, Forell, Oliveira, & Rodrigues, 2002). Freezing, on the other hand, did not influence sperm viability, which remained very high (86%), indicating the high quality of the semen. However, the most relevant change recorded after freezing was the percentage of DNA-fragmented sperm that increased by 14% compared to fresh semen. It is worth underlining that DNA fragmentation index has been previously negatively associated with sperm fertility outcome (Anzar et al., 2002; García-Macías et al., 2006). An increased DNA fragmentation in cryopreserved sperm, detected by Tunel, was also reported in an earlier work (Takeda, Uchiyama, Kinukawa, Tagami, Kaneda, & Watanabe, 2015). This finding is, however, in contrast with another previous work that reported Δψm decrease, caspase activation and permeability membrane increase that are typical apoptotic features, following semen cryopreservation, with no effect on sperm DNA fragmentation (Martin et al., 2004). Contradicting results may be accounted for by differences in cryopreservation procedure, including extender composition, among studies. It is known that DNA fragmentation is a final event of apoptosis, triggered by the activation of caspases, that occurs during the degradation phase of the apoptotic pathway, resulting from mitochondrial destabilization and subsequent activation of pro-apoptotic factors (Nagata, Nagase, Kawane, Mukae, & Fukuyama, 2003; Scovassi & Torriglia, 2003).

The main objective of this study was to assess whether inhibiting caspase activation with Z-VAD-FMK, before and after freezing, would reduce cryopreservation-induced apoptosis and hence improve the qualitative characteristics of bovine frozen-thawed semen. For this purpose, two different concentrations of Z-VAD-FMK (20 and 100 μM), based on literature (Pero et al., 2018; Peter et al., 2005; Peter & Linde-Forsberg, 2003), were tested. The impact of the apoptotic inhibitor on sperm was examined in terms of motility, viability, membrane integrity, DNA fragmentation and mitochondrial membrane potential ($\Delta\psi_m$). Our results in part confirmed our initial hypothesis, as the use of the caspase inhibitor Z-VAD-FMK was effective in preventing apoptosis induced by freezing/thawing, as indicated by the decreased percentage of DNA-fragmented sperm in semen treated with 100 μM Z-VAD-FMK both before and after cryopreservation. The enrichment of the extender with Z-VAD-FMK, especially at the highest concentration, increased post-thawing mass motility, reduced the DNA fragmentation index, with a tendency to increase the mitochondrial membrane potential. With regard to the latter parameter, however, it is worth noting that the effect was different according to the dose. A significant reduction in the percentage of DNA-fragmented sperm, as well as an increase in intact-membrane spermatozoa, was also observed when the semen was treated for 1 hr after thawing with the highest concentration of Z-VAD-FMK. However, the treatment was uninfluential on motility, viability and $\Delta\psi_m$. When sperm was treated both before and

TABLE 3 Sperm fertility parameters in groups treated with 0, 20 μM and 100 μM Z-VAD Z-VAD for 1 hr after thawing (4 ejaculates per 12 bulls)

	Treatments		
Groups	0 (control) Mean ± *SE*	20 μM Z-VAD Mean ± *SE*	100 μM Z-VAD Mean ± *SE*
Motility (%)	60.0 ± 5.8	62.5 ± 7.2	67.5 ± 4.3
Viability (%)	82.3 ± 0.5	84.6 ± 1.0	84.3 ± 2.1
HOS+ (%)	60.5 ± 3.5^{Aa}	70.9 ± 1.2^{b}	74.3 ± 2.1^{B}
ΔΨm (%)	62.4 ± 12.7	57.9 ± 12.8	50.8 ± 8.8
Tunel+ (%)	17.8 ± 1.1^{a}	13.3 ± 2.8^{ab}	10.5 ± 2.5^{b}

Note: Values within rows with different superscript uppercase letters are significantly different; $p < .01$.

Values within rows with different superscript lowercase letters are significantly different; $p < .05$.

after with the caspase inhibitor, no additional benefit was observed (data not shown).

These results should be regarded with caution, since the variations detected in sperm parameters are variable and not conclusive. Undoubtedly, the treatment with Z-VAD- FMK resulted in a decrease of sperm DNA fragmentation, both when the treatment was applied before and after freezing. The reduced DNA fragmentation may result from the inhibition of caspases by Z-VAD-FMK. It was previously demonstrated that cryopreservation is associated with caspase activation in bovine sperm (Martin et al., 2004). Z-VAD-FMK is known to bind to the catalytic sites of caspases 8 and 9 (initiators) and caspase 3 (effector), preventing their activation. The inactive pro-caspases are present in most cells including pre-implantation embryo (Jurisicova, Antenos, Varmuza, Tilly, & Casper, 2003; Warner et al., 1998) and gametes (Jurisicova et al., 2003; Martin et al., 2007) and their activation in response to many apoptotic triggers, results in dismantling cytoskeleton components, as well as activation of DNA degrading endonucleases (van Loo et al., 2001), leading to DNA fragmentation. Several studies demonstrated that apoptosis can be prevented by inhibiting caspase activity, resulting in increased cryosurvival in mammalian cells (Stroh et al., 2002; Yagi et al., 2001), embryos (Pero et al., 2018) and oocytes (Wasielak & Bogacki, 2007). Our results are in agreement with recent studies that reported reduced apoptosis-like changes during cryopreservation of buffalo semen after treatment with caspase inhibitors (Dalal et al., 2019, 2018). In contrast, the addition of caspase inhibitors in the extender failed to improve cryotolerance in ram, dog and stallion sperm (Marti, Perez-Pe, & Colas, 2008; Peter et al., 2005; Peter & Linde-Forsberg, 2003).

In conclusion, the treatment of bovine semen with 100 μM of the caspase inhibitor Z-VAD-FMK before freezing increased sperm mass motility and ΔΨm, while decreasing sperm DNA fragmentation. Treatment of semen after thawing with 100 μM Z-VAD-FMK resulted in improved sperm membrane integrity and reduced DNA fragmentation. Therefore, inhibiting caspase activity both before and after freezing led to a reduction in sperm showing fragmented DNA, suggesting that this treatment is efficient to prevent cryopreservation-induced apoptosis. However, as the treatment did not improve all the quality parameters examined, further studies are needed to assess the sperm fertilizing ability before suggesting the potential use of Z-VAD-FMK for sperm preservation in cattle.

CONFLICT OF INTEREST

The authors declare there is no conflict of interest that would prejudice the impartiality of this scientific work.

AUTHOR CONTRIBUTIONS

NP, VL, CDC, KMA participated in laboratory analysis, in acquisition of biological material, interpretation of the data and drafting the article. CZ and AR participated in laboratory analysis (flow cytometer) and interpretation of the data. MEP and BG participated in the conception and design of the study, interpretation of the data, statistical analysis, drafting and final approval of the submission.

DATA AVAILABILITY

All data relevant to the study are included in the article.

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REFERENCES

Anzar, M., He, L., Buhr, M. M., Kroetsch, T. G., & Pauls, K. P. (2002). Sperm apoptosis in fresh and cryopreserved bull semen detected by flow cytometry and its relationship with fertility. *Biology of Reproduction*, *66*, 354–360. https://doi.org/10.1095/biolreprod66.2.354

Aziz, N., Said, T., Paasch, U., & Agarwal, A. (2007). The relationship between human sperm apoptosis, morphology and the sperm deformity index. *Human Reproduction*, *22*, 1413–1419. https://doi.org/10.1093/humrep/dem016

Aziz, N., Saleh, R. A., Sharma, R. K., Lewis-Jones, I., Esfandiari, N., Thomas, A. J., & Agarwal, A. (2004). Novel association between sperm reactive oxygen species production, sperm morphological defects, and the sperm deformity index. *Fertility and Sterility*, *81*, 349–354. https://doi.org/10.1016/j.fertnstert.2003.06.026

Baust, J. M., Van Buskirk, R., & Baust, J. G. (2000). Cell viability improves following inhibition of cryopreservation-induced apoptosis. *Vitro Cellular & Developmental Biology - Animal*, *36*(4), 262–270. https://doi.org/10.1290/1071-2690(2000)036<0262:CVIFIO>2.0.CO;2

Boccia, L., Di Palo, R., De Rosa, A., Attanasio, L., Mariotti, E., & Gasparrini, B. (2007). Evaluation of buffalo semen by Trypan blue/Giemsa staining and related fertility in vitro. *Italian Journal of Animal Science*, *6*(2), 739–742. https://doi.org/10.4081/ijas.2007.s2.739.

Bratton, D. L., Fadok, V. A., Richter, D. A., Kailey, J. M., Guthrie, L. A., & Henson, P. M. (1997). Appearance of phosphatidylserine on apoptotic cells requires calcium-mediated nonspecific flip-flop and is enhanced by loss of the aminophospholipid translocase. *Journal of Biological Chemistry*, *272*, 26159–26165. https://doi.org/10.1074/jbc.272.42.26159

Dalal, J., Kumar, A., Honparkhe, M., & Brar, P. S. (2019). Effect of Z-IETD-Fmk (Caspase Inhibitor) supplementation on apoptosis like changes developed in buffalo bull sperm during cryopreservation. *International Journal of Current Microbiology and Applied Sciences*, *8*(02), 516–524. https://doi.org/10.20546/ijcmas.2019.802.059

Dalal, J., Kumar, A., Honparkhe, M., Singh, A. K., & Brar, P. S. (2018). Minimization of apoptosis like changes developed during cryopreservation of buffalo bull sperm by supplementing Z-LEHD-FMK (Caspase Inhibitor). *International Journal of Current Microbiology and Applied Sciences*, *7*(09), 1076–1084. https://doi.org/10.20546/ijcmas.2018.707.388

García-Macías, V., de Paz, P., Martinez-Pastor, F., Álvarez, M., Gomes-Alves, S., Bernardo, J., … Anel, L. (2006). DNA fragmentation assessment by flow cytometry and Sperm-Bos-Halomax (bright-field microscopy and fluorescence microscopy) in bull sperm. *International Journal of Andrology*, *30*(2), 88–98. https://doi.org/10.1111/j.1365-2605.2006.00723.x

Hendricks, K. E., & Hansen, P. J. (2009). Can programmed cell death be induced in post-ejaculatory bull and stallion spermatozoa? *Theriogenology*, *71*, 1138–1146. https://doi.org/10.1016/j.theriogenology.2008.12.006.

Jurisicova, A., Antenos, M., Varmuza, S., Tilly, J. L., & Casper, R. F. (2003). Expression of apoptosis-related genes during human preimplantation embryo development: Potential roles for the harakiri gene product and caspase-3 in blastomere fragmentation. *Molecular Human Reproduction*, *9*, 133–141. https://doi.org/10.1093/molehr/gag016

Jeyendran, R. S., Van der Ven, H. H., Perez-Pelaez, M., Crabo, B. G., & Zaneveld, L. J. (1984). Development of an assay to assess the

functional integrity of the human sperm membrane and its relationship to other semen characteristics. *Journal of Reproduction and Fertility*, *70*(1), 219–28. https://doi.org/10.1530/jrf.0.0700219

Marti, E., Perez-Pe, R., & Colas, C. (2008). Study of apoptosis-related markers in ram spermatozoa. *Animal Reproduction Science*, *106*,113–132. https://doi.org/10.2164/jandrol.107.003459

Martin, G., Cagnon, N., Sabido, O., Sion, B., Grizard, G., Durand, P., & Levy, R. (2007). Kinetics of occurrence of some features of apoptosis during the cryopreservation process of bovine spermatozoa. *Human Reproducyion*, *22*(2), 380–388. https://doi.org/10.1093/humrep/del399

Longobardi, V., Zullo, G., Salzano, A., De Canditiis, C., Cammarano, A., De Luise, L., Gasparrini, B. (2017). Resveratrol prevents capacitation-like changes and improves in vitro fertilizing capability of buffalo frozen-thawed sperm. *Theriogenology.*, *88*, 1–8. https://doi.org/10.1016/j.theriogenology.2016.09.046.

Martin, G., Sabido, O., Durand, P., & Levy, R. (2004). Cryopreservation induces an apoptosis-like mechanism in bull sperm. *Biology of Reproduction*, *71*(1), 28–37. https://doi.org/10.1095/biolreprod.103.024281

Medeiros, C. M., Forell, F., Oliveira, A. T., & Rodrigues, J. L. (2002). Current status of sperm cryopreservation: why isn't it better? *Theriogenology*, *57*(1), 327–344. https://doi.org/10.1016/S0093-691X(01)00674-4

Men, H., Monson, R. L., Parrish, J. J., & Rutledge, J. J. (2003). Degeneration of cryopreserved bovine oocytes via apoptosis during subsequent culture. *Cryobiology*, *47*, 73–81. https://doi.org/10.1016/s0011-2240(03)00070-1

Men, H., Agca, Y., Riley, L. K., & Critser, J. K. (2006). Improved survival of vitrified porcine embryos after partial delipation through chemically stimulated lipolysis and inhibition of apoptosis. *Theriogenology*, *66*(8), 2008–2016. https://doi.org/10.1016/j.theriogenology.2006.05.018

Nagata, S., Nagase, H., Kawane, K., Mukae, N., & Fukuyama, H. (2003). Degradation of chromosomal DNA during apoptosis. *Cell Death & Differentiation*, *10*(1), 108–116. https://doi.org/10.1038/sj.cdd.4401161

Niu, Y., Jianjun, D., Chen, Y., Wu, C., Zhang, S., & Zhang, D. (2016). Positive effect of apoptotic inhibitor Z-VAD-FMK on vitrified-thawed porcine MII stage oocytes. *Cryoletters*, *37*(3), 188–195.

Ortega-Ferrusola, C., Sotillo-Galán, Y., Varela-Fernández, E., Gallardo-Bolaños, J. M., Muriel, A., González-Fernández, L., … Peña, F. J. (2008). Detection of "Apoptosis-Like" Changes During the Cryopreservation Process in Equine Sperm. *Journal of Andrology*, *29*, 213–221. https://doi.org/10.2164/jandrol.107.003640.

Paasch, U., Sharma, R. K., Gupta, A. K., Grunewarld, S., Mascha, E. J., Thomas, A. J., … Agarwal, A. (2004). Cryopreservation and thawing is associated with varying extent of activation of apoptotic machinery in subsets of ejaculated human spermatozoa. *Biology of Reproduction*, *71*, 1828–1837. https://doi.org/10.1095/biolreprod.103.025627

Parks, J. E., & Graham, J. K. (1992). Effects of cryopreservation procedures on sperm membranes. *Theriogenology*, *38*, 209–222. https://doi.org/10.1016/0093-691x(92)90231-f

Parrish, J. J., Susko-Parrish, J. L., Leibfried-Rutledge, M. L., Critser, E. S., Eyestone, W. H., & First, N. (1986). Bovine in vitro fertilization with frozen-thawed semen. *Theriogenology.*, *25*, 591–600. https://doi.org/10.1016/0093-691x(86)90143-3

Peña, F. J., Johannisson, A., Wallgren, M., & Rodríguez-Martínez, H. (2003). Assessment of fresh and frozen-thawed boar semen using annexin-V assay: A new method of evaluating sperm membrane integrity. *Theriogenology*, *60*, 677–689. https://doi.org/10.1016/s0093-691x(03)00081-5

Pero, M. E., Lombardi, P., Longobardi, V., Boccia, L., Vassalotti, G., Zicarelli, L., … Gasparrini, B. (2017). Influence of γ-glutamyltransferase and alkaline phosphatase activity on in vitro fertilization of bovine frozen/thawed semen. *Italian Journal of Animal Science*, *16*(03), 390–392. https://doi.org/10.1080/1828051X.2017.1290509

Pegg, D. E. (2015). Principles of cryopreservation. In W. F. Wolkers, & H. Oldenhof (Eds.), *Cryopreservation and Freeze-Drying Protocols*, *3rd edn*, *Methods in Molecular Biology 1257* (pp. 3–19). New York: Springer.

Pero, M. E., Zullo, G., Esposito, L., Iannuzzi, A., Lombardi, P., De Candidis, C., … Gasparrini, B. (2018). Inhibition of apoptosis by caspase inhibitor Z-VAD-FMK improves cryotolerance of in vitro derived bovine embryos. *Theriogenology*, *108*, 127–135. https://doi.org/10.1016/j.theriogenology.2017.11.031

Peter, A. T., Colenbrander, B., & Gadella, B. M. (2005). Effect of caspase inhibitors on the post-thaw motility, and integrity of acrosome and plasma membrane of cryopreserved equine spermatozoa. *Indian Journal Experimental Biology*, *43*(6), 483–487.

Peter, A. T., & Linde-Forsberg, C. (2003). Efficacy of the anticaspase agent zVAD-fmk on post-thaw viability of canine spermatozoa. *Theriogenology*, *59*, 1525–1532. https://doi.org/10.1016/S0093-691X(02)01206-2

Ravagnan, L., Roumier, T., & Kroemer, G. (2002). Mitochondria, the killer organelles and their weapons. *Journal of Cellular Physiolology*, *192*, 131–137. https://doi.org/10.1002/jcp.10111

Scovassi, A. I., & Torriglia, A. (2003). Activation of DNA-degrading enzymes during apoptosis. *European Journal of Histochemistry*, *47*, 185–194.

Stroh, C., Cassens, U., Samraj, A. K., Sibrowski, W., Schulze-Osthoff, K., & Los, M. (2002). The role of caspases in cryoinjury: Caspase inhibition strongly improves the recovery of cryopreserved hematopoietic and other cells. *Federation American Societies for Experimental Biology*, *16*, 1651–1653. https://doi.org/10.1096/fj.02-0034fje

Takeda, K., Uchiyama, K., Kinukawa, M., Tagami, T., Kaneda, M., & Watanabe, S. (2015). Evaluation of sperm DNA damage in bulls by TUNEL assay as a parameter of semen quality. *Journal of Reproduction and Devopment*, *61*, 185–190. https://doi.org/10.1262/jrd.2014-140.

van Loo, G., Schotte, P., van Gurp, M., Demol, H., Hoorelbeke, B., Gevaert, K., … Vandenabeele, P. (2001). Endonuclease G: A mitochondrial protein released in apoptosis and involved in caspase-independent DNA degradation. *Cell Death and Differentiation*, *8*(12), 1136–1142. https://doi.org/10.1038/sj.cdd.4400944

Warner, C. M., Cao, W., Exley, G. E., McElhinny, A. S., Alikani, M., Cohen, J., … Brenner, C. A. (1998). Genetic regulation of egg and embryo survival. *Human Reproduction*, *13*, 178–190. https://doi.org/10.1093/humrep/13.suppl_3.178

Wasielak, M., & Bogacki, M. (2007). Apoptosis inhibition by insulin-like growth factor (IGF)-I during in vitro maturation of bovine oocytes. *The Journal of Reproduction and Development*, *53*(2), 419–426. https://doi.org/10.1262/jrd.18076

Woods, E. J., Benson, J. D., Agca, Y., & Critser, J. K. (2004). Fundamental cryobiology of reproductive cells and tissues. *Cryobiology*, *48*, 146–156.https://10.1016/j.cryobiol.2004.03.002.

Wyllie, A. H., Kerr, J. F. R., & Currie, A. R. (1980). Cell death: The significance of apoptosis. *International Review of Cytology*, *68*, 251–306.

Yagi, T., Hardin, J. A., Valenzuela, Y. M., Miyoshi, H., Gores, G. J., & Nyberg, S. L. (2001). Caspase inhibition reduces apoptotic death of cryopreserved porcine hepatocytes. *Hepatology*, *33*, 1432–1440. https://doi.org/10.1053/jhep.2001.24560

Zhivotovsky, B., Burgess, D. H., Vanags, D. M., & Orrenius, S. (1997). Involvement of cellular proteolytic machinery in apoptosis. *Biochemical and Biophysical Research Communication*, *230*, 481–488. https://doi.org/10.1006/bbrc.1996.6016.

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