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Minimization of Apoptosis like Changes by Supplementing Z-DEVD-FMK (CASPASE inhibitor) during Cryopreservation of Buffalo Bull Semen

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A B S T R A C T

Present study was conducted to evaluate the anti-apoptotic effect of caspase inhibitor (Z-DEVD-FMK) supplementation in buffalo bull semen. Z-DEVD-FMK was supplemented with Tris egg yolk extender @ 2, 4, 6, 10, 20 µM. Pre-freeze and post thaw samples were evaluated in terms of % individual motility, % viability, % HOS reactive sperms, status of mitochondrial membrane potential and status of sperm membrane phosphatidylserine. There was no significant effect after supplementation of caspase inhibitor on % sperm motility, % active mitochondria and % sperms with low PLA activity in pre-freeze semen samples. Supplementation of Z-DEVD-FMK did not improve sperm motility in post freeze semen sample also. However, there was significant ($P < 0.05$) improvement in terms of % live sperm, % HOS reactive Sperms, % active mitochondria and % sperms with low PLA activity in all supplementation doses of Z-DEVD-FMK in post freeze semen samples as compared to control (without supplementation). The percent live sperms and HOS reactive sperms were significantly ($P < 0.05$) higher @ 2 µM supplementation doses of Z-DEVD-FMK (i.e75.23% and 72.5%) as compared to other supplementation doses @ 4 µM (62.55% and 60.34%), 6 µM (60.38% and 58.76%), 10 µM (58.56% and 59.5%), 20 µM (61.36% and 60.56%) and control (58.8% and 50.4%). Live sperms and HOS reactive were significantly lower ($P > 0.05$) (46.36% and 44.56%) @ 20 µM as compared to control. However, % active mitochondria and % sperms with low PLA activity was significantly ($P < 0.05$) higher @ 20µM supplementation doses of Z-DEVD-FMK as compared to other supplementation doses (2µM, 4 µM, 6µM, 10µM) and control.

Introduction

Artificial insemination with cryopreserved semen is a widely used technique in buffalo (Singh and Balhara, 2016). However, the fertility of cryopreserved semen remains poor (33%) as compared to fresh semen (Chohan *et al.,* 1992). One of the reasons for poor fertility of cryopreserved semen is freezing induced apoptosis like changes inflicted in spermatozoa indicated by externalization of phosphatidylserine (PS) due to higher

phospholipase activity (PLA) (Glander *et al.,* 2002). The improvement in post thaw semen quality could be done by minimizing apoptosis like changes developed during cryopreservation. Apoptosis-like changes has been identified by the presence of caspase 9 and caspase 3 in bovine semen (Anzar *et al.,* 2002), increased membrane permeability and decreased mitochondrial membrane potential in equine semen (Ferrusola *et al.,* 2008).

Martin *et al.,* (2004) found that, after cryopreservation, majority of living sperm cells showed low mitochondrial potential. The caspases activates DNAase and are responsible for DNA fragmentation (Enari *et al.,* 1998) and in return, DNA damage can also initiate apoptosis (Danial and Korsmeyer, 2004). Apoptotic sperm with fragmented DNA and damaged membrane results in poor fertility rates (Erickson *et al.,* 2015). Caspases are synthesized as inactive proenzyme (pro-caspases) which are activated by cleavage during the cascade of ordered events of apoptosis (Cohen, 1997).

The existence of caspase-dependent apoptotic-like mechanisms associated with mitochondrial functionality in sperm, possibly similar to those found in somatic cells (Boise and Thompson, 1997; Ricci *et al.,* 2003, 2004; Lakhani *et al.,* 2006). Z-DEVD-FMK inhibits caspase 3 and 7 (Alicia *et al.,* 2006) and Caspase 3 and caspase 7 are well known executioner of apoptosis pathways (Vilmont *et al.,* 2012). Z-DEVD-FMK (Sigma) is a cell-permeable inhibitor of caspase-3, -6, -7, -8, and -10 (Pichardo *et al.,* 2010).

Caspases such as the effector caspase-7 and the caspase-10 are involved in the membrane pathway of apoptosis (Slee *et al.,* 1999). So, supplementation of caspase inhibitor, Z-DEVD-FMK could be of use in minimizing apoptosis like changes (Alicia *et al.,* 2006).

Materials and Methods

Ethical approval

The approval from the institutional animal ethics committee to carry out the present study was not required as it did not involve handling of live animals and no invasive technique was used. Semen was being collected and frozen as a routine procedure under progeny testing program.

Selection of buffalo bulls

Three breeding buffalo bull around 4 yrs of age maintained at bull farm, GADVASU, Punjab, India (Latitude/Longitude, 30.55°N, 75.54° E) was included in the present study. These bulls were under progeny testing program and were being used for semen collection by artificial vagina method. Bulls were maintained under loose housing system (covered area - 12 x 10 ft and uncovered area - 25 x 10 ft) and standard feeding schedule along with adlib green fodder.

Experimental design

Five ejaculates from each buffalo bulls were used in this study. Each ejaculate was extended with Tris egg yolk extender. Each ejaculate was supplemented with Z-DEVD-FMK in five concentrations (@ 2, 4, 6, 10 and 20 µM). Each ejaculate extended in Tris egg yolk extender and from these 6 aliquots were taken. Out of these 6 aliquots, 5 were used for supplementation of Z- DEVD-FMK and one was kept as control i.e. without supplementation. Caspase inhibitor was dissolved in dimethyl sulphoxide (DMSO) to achieve desire concentration. Semen samples were frozen using traditional vapour freezing method. The quality of pre-freeze and post thaw semen in terms of % individual motility, % viability, % HOST reactive sperms, % active mitochondria and % sperm with low

PLA activity (non-apoptotic sperms) were evaluated.

The % individual motility was assessed manually under 20 x objective of phase contrast microscope (Nikon Eclipse E 200). The live sperm count was determined through Eosin-Nigrosin staining technique as per standard procedure (Blom *et al.,* 1977). The HOS test was performed as per standard procedure to assess the functional integrity of sperm membrane (Jeyendran *et al.,* 1984)).

Evaluation of mitochondrial membrane potential in caspase inhibitors supplemented pre-freeze and post thaw semen

Mitochondrial membrane potential was assessed by using fluorescent dye Tetra-methylrhodamine, methyl ester (TMRM, Life Technologies; Cat#T-668) as reported in previous study (Dalal *et al.,* 2016). Briefly, semen samples (pre-freeze and post thaw; 250µl) were given 2 washings with PBS by centrifuging at 1000 RPM for 5 min at 37^0C. Then, 5 µl of 50 µM TMRM solution in DMSO was added to each sample and incubated at 37^0C for 90 min. After incubation, washing was done with PBS at 1000 RPM for 5 min at 37^0C to remove all the unbound dye. The sperm pellet was mixed well with 500 µl of PBS. On a microslide, 10 µl of washed sample and 8 µl of ProLong Gold Antifade Mountant with DAPI (Life Technologies, Cat# P36941) was taken and covered with coverslip.

The slide was examined under upright fluorescent microscope (Nikon) with DAPI filter (420-480 nm), FITC FILTER (510 - 580nm) and TRITC filter (530-580nm). Around 100 sperms were observed for high or low fluorescence in mid piece region as an indicator of mitochondrial membrane potential.

Evaluation of sperm phospholipase activity in caspase inhibitors supplemented pre-freeze and post thaw semen

Sperm phospholipid membrane was studied using BODIPY C11 fluorescent dye (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (BODIPY C11 FL, Life technologies, Cat# D 3862) as reported in previous study (Dalal *et al.,* 2016). Briefly, semen samples (pre-freeze and post thaw; 250 µl) were given 2 washings with PBS by centrifuging at 1000 RPM for 5 min at 37^0C. Then, 30 µl of 20 µM BODIPY solution in DMSO was added to each semen sample and incubated for 45 min at 37 ^{0}C. After incubation, washing was done with 1ml of PBS at 1000 RPM for 5 min at 37^0C to remove all the unbound dye. The pellet was mixed well with 500 µl of PBS. On a micro slide, 10µl of sample and 8 µl of ProLong Gold Antifade Mountant with DAPI (Life Technologies, Cat# P36941) was taken and covered with cover slip. Glass slides were examined under upright fluorescent microscope (Nikon) with DAPI filter (420-480 nm), FITC filter (510 - 580nm) and TRITC filter (530-580nm). Around 100 sperms in different fields were observed and normal sperm without fluorescence were calculated out of hundred and taken as % sperm with low PLA (phospholipase A1 and A2) activity.

The data was analyzed for one-way analysis of variance (ANOVA) and Games Howell Post hoc test using IBM SPSS Version 20.

Results and Discussion

In our study, Tris extender was supplemented with Z-DEVD-FMK (caspase inhibitor) in the final concentration at 2, 4, 6, 10, and 10 µM and evaluated the pre-freeze and post-thaw semen samples in terms of percent individual motility, viability, HOST reactive sperms, mitochondrial membrane activity, and sperm

PLA activity status. Data obtained was analyzed and presented in Table 1.

Sperm motility

There was no significant (P > 0.05) difference in terms of % motility in pre-freeze supplementation groups and control as shown in Table 1. Post thaw % motility in supplemented doses (2, 4, 6, 10 and 20 µM) and control were similar (P > 0.05) as shown in Table 1. The mechanisms of inducing apoptosis by different caspases are more complex and many factors are involved (Sule *et al.,* 2013). In our study, Z-DEVD-FMK did not affect the % motility. Chen *et al.,* (2006) reported the inverse relationship between sperm motility and apoptosis in human spermatozoa.

Sperm viability

There was no significant (P > 0.05) difference in % sperm viability in pre-freeze supplementation groups and control group (Table 1). However, in post thaw samples, % viable sperms were significantly (P < 0.05) higher in a group with 2 µM (75.23±4.66) of Z-DEVD-FMK supplementation as compared to other supplemented doses 4 (62.55±3.56), 6 (50.38±4.64), 10 (52.56±5.11) and 20 µM (46.36±3.99) and control (50.8). Percent viable sperms post thaw samples were similar (P > 0.05) between 6 (50.38±4.64), 10 µM (52.56±5.11) of Z-DEVD-FMK supplementation and control (50.8), however these were significantly (P < 0.05) higher than % viability obtained at 20 µM (46.36±3.99) of Z-DEVD-FMK supplementation.

Table.1 Effects of supplementation of Z-DEVD-FMK at various concentrations at pre-freeze and post thaw stage

Parameters	Pre-freeze						Post thaw					
	Control	**2 µM**	**4 µM**	**6 µM**	**10 µM**	**20 µM**	**Control**	**2 µM**	**4 µM**	**6 µM**	**10 µM**	**20 µM**
Motility	85 ± 4.06	90 ± 5.23	85 ± 3.99	85 ± 4.65	85 ± 5.87	85 ± 4.87	45.5 ± 4.33 ^{a}	40.25 ± 2.38^{a}	38.41 ± 3.76 ^{a}	42.0 ± 3.56 ^{a}	43.78 ± 5.49 ^{a}	45.67 ± 5.77 ^{a}
% Viability	90.19± 4.62	92.8± 5.5	96± 4.78	88.82± 5.67	88.8± 5.12	94.59± 5.45	50.8± 4.65 ^{a}	75.23± 4.66 ^{b}	62.55± 3.56 ^{c}	50.38± 4.64 ^{a}	52.56± 5.11 ^{a}	46.36± 3.99 ^{d}
% Active mitochondria	86.20 ± 4.23	85.22 ± 3.80	87.23 ± 2.22	90.45 ± 4.21	85.54 ± 2.32	88.8 ± 3.22	65.6 ± 3.45 ^{a}	78.8 ± 3.45 ^{b}	75.4 ± 2.33 ^{b}	77.5 ± 1.45 ^{b}	82.6 ± 3.81^{bd}	85.4 ± 3.22^{bde}
% HOS reactive sperm	82.76± 5.81	78.2± 4.87	73± 6.77	77± 5.44	76± 6.87	65± 5.98	50.4± 3.87 ^{a}	68.5± 4.16 ^{b}	60.34± 5.98 ^{c}	56.76± 5.43 ^{c}	58.5± 4.12^{c}	44.56± 4.55^{d}
% sperms with low PLA activity	80.45 ± 4.23	78.48 ± 4.55	83.44 ± 2.89	85.77 ± 5.76	85.12 ± 4.66	85.67 ± 3.42	60.87 ± 4.68 ^{a}	74.11 ± 4.75^{b}	78.55 ± 3.11^{b}	73.36 ± 5.32^{b}	75.45 ± 6.56 ^{b}	80.51 ± 5.55 ^{b}

Values marked with dissimilar superscript differ significantly (P < 0.05) within a row

Mitochondrial status

In pre-freeze samples, there were no significant (P > 0.05) differences between control (without Z-DEVD-FMK) and supplemented (2, 4, 6, 10 and 20 µM doses of Z-DEVD-FMK) in terms of % active mitochondria.

In post thaw samples, the % active mitochondria were significantly (P < 0.05) higher with supplementation of Z-DEVD-FMK @ 2 µM (78.8 ± 3.45), 4 µM (75.4 ± 2.33), 6 µM (77.5 ± 1.45), 10 µM (82.6 ± 3.81) and 20 µM (85.4 ± 3.22) as compared to control (65.6 ± 3.45). The % active mitochondria in post thaw samples with supplementations @ 2 µM (78.8 ± 3.45), 4 µM (75.4 ± 2.33) and 6 µM (77.5 ± 1.45) doses were similar (P > 0.05). But, % active mitochondria in 10 µM (82.6 ± 3.81) were significantly higher as compared to 2 µM (78.8 ± 3.45), 4 µM (75.4 ± 2.33) and 6 µM (77.5 ± 1.45) doses. However, Supplementation of Z-DEVD-FMK @ 20µM (85.4 ± 3.22) resulted in significantly (P < 0.05) higher % active mitochondria as compared to other supplementation doses. Hence, supplementation of Z-DEVD-FMK helped in maintaining mitochondrial membrane potential of spermatozoa following cryopreservation of semen.

PLA activity

In pre-freeze samples, there were no significant (P < 0.05) difference between control and Z-DEVD-FMK supplemented doses (2, 4, 6, 10 and 20 µM) in terms of % sperms with low PLA activity. The % sperms with low PLA activity in post thaw samples were significantly (P < 0.05) higher @ 2 µM (74.11 ± 4.75), 4 µM (78.55 ± 3.11), 6 µM (73.36 ± 5.32), 10 µM (75.45 ± 6.56) and 20 µM (80.51 ± 5.55) doses of Z-DEVD-FMK as compared to control (60.87 ± 4.68). There were no significant differences in % sperms with low PLA activity among all supplementation doses of Z-DEVD-FMK. Our study indicated Z-DEVD-FMK supplementation has protective effect against apoptosis on spermatozoa during cryopreservation.

Hypo osmotic swelling test (HOST)

In pre-freeze samples, there were no significant (P < 0.05) difference between control and Z-DEVD-FMK supplemented doses (2, 4, 6, 10 and 20 µM) in terms of % Host reactive sperms. The % Host reactive sperms in post thaw samples were significantly (P<0.05) higher @ 2 µM (68.5±4.16), 4 µM (60.34±5.98), 6 µM (56.76±5.43), 10 µM (58.5±4.12) and significantly (P>0.05) lower @ 20 µM (44.56±4.55) doses of Z-DEVD-FMK as compared to control (50.4±3.87). The % Host reactive sperms in post thaw samples were significantly (P<0.05) higher @ 2 µM (75.23±4.66) as compared to 4 µM (60.34±5.98), 6 µM (56.76±5.43), 10 µM (58.5±4.12) and 20 µM (44.56±4.55). The % Host reactive sperms in post thaw samples were significantly (P<0.05) higher @ 4 µM (60.34±5.98), 6 µM (56.76±5.43), 10 µM (58.5±4.12) as compared to 20 µM (44.56±4.55).

There are very few reports available in literature related to this aspect and most of these are of human spermatozoa. The increase in caspase activation is dependent on the applied sperm preparation and cryopreservation protocol (Grunewald *et al.*, 2005d). Cryopreservation has been reported to activate caspase-3 and -9 in man (Paasch *et al.*, 2004; Bejarano *et al.*, 2008) and activated caspase-3 has been found in man (Choi *et al.*, 2008;) and boar (van Gurp *et al.*, 2003). Caspase activation following the cryopreservation and thawing process was

also seen in bovine (Martin *et al.*, 2004, 2007) and equine spermatozoa (Brum *et al.*, 2008; Ferrusola *et al.*, 2008). The highest cryopreservation-induced increase in caspase activation was found in human sperm positive foractive Caspase-3 (32.6%) followed by active Caspase-8 sperm (30.5%), active Caspase-9 sperm (22.2%) and active Caspase-11 sperm (15.5%) underlining the central role of the effector caspase-3 (Paasch *et al.*, 2004). Hence caspase-3 marks a ‘‘point of no return’’ in the apoptosis signaling cascade, the pronounced activation of the protease by cryopreservation and thawing displays the deleterious influence of this process on human sperm. Therefore, monitoring caspase-3 activation should be used for further optimization of cryopreservation and thawing protocols. Procaspase-9 was present in bull sperm, but procaspase-3 and -8 were absent and cryopreservation has been reported to activate caspase-9 in bull sperm (Martin *et al.*, 2007). It is well known fact that Z-DEVD-FMK (Sigma) is a cell-permeable inhibitor of caspase-3, -6, -7, -8, and -10 (Pichardo *et al.*, 2010). Our study indicates that Z-DEVD-FMK supplementation improves the mitochondrial potential and % sperms with low PLA activity in dose dependent manner. However, it has been reported that the addition of caspase inhibitors to the cryopreservation medium failed to improve the acrosome and plasma membrane integrity of frozen-thawed ram (Marti *et al.*, 2008), dog (Peter and Linde-Forsberg, 2003), and stallion spermatozoa (Peter *et al.*, 2005). These differences from our study may be due tothespecies difference or difference in doses of supplementation. Peter *et al.*, (2005) also suggested that a higher or lower level of caspase doses and different timing of treatment may produce the desired effects. However, Marti *et al.*, (2008) reported that the addition of z-VAD-fmk to the capacitation medium counteracted the PS inversion due to capacitation as the percentage of intact ram spermatozoa (without PS inversion) increased ($P < 0.05$) up to the same value of control samples (50.3±2.0 versus 37.0±3.7).

In conclusion, the Z-DEVD-FMK supplementation improves the mitochondrial potential and % sperms with low PLA activity in post thaw semen samples in dose dependent manner. There was improvement following Z-DEVD-FMK supplementation in terms of % viability and Host reactive sperm in post thaw samples at lower doses (2, 4 and 6µM) whereas at higher dose level (20 µM) there was no improvement in these parameters. It implies that apoptotic like changes were developed during cryopreservation and Z-DEVD-FMK supplementation help to counteract development of these apoptosis like changes in sperm.

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