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Minimization of Apoptosis like Changes Developed during Cryopreservation of Buffalo Bull Sperm by Supplementing Z-LEHD-FMK (Caspase Inhibitor)

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A B S T R A C T

Present study was undertaken to evaluate the anti-apoptotic effect of LEHD-FMK (caspase inhibitor) on buffalo bull sperm before and after semen freezing. Tris egg yolk extender was treated with Z-LEHD-FMK (0, 2, 4, 6, 10 and 20 µM) and used for extension of six aliquots of semen from each ejaculate to final concentration of 80 million sperm/ml. Sperm samples were analyzed for sperm motility, viability, plasma membrane integrity, mitochondrial membrane potential and PLA (Phospholipase) activity at pre-freeze and post thaw stages. There was no effect ($P>0.05$) of this caspase inhibitor on percent sperm motility, viability, plasma membrane integrity, active mitochondria and sperms with low PLA (Phospholipase) activity before semen freezing. However, Z-LEHD-FMK (2µM) treated sperm showed higher ($P<0.05$) motility with functionally intact membrane at post thaw stage as compared to control (0 µM). Percent post thaw viable sperm were higher ($P<0.05$) in Z-LEHD-FMK (2, 4 and 6 µM) treated semen sample as compared to control. Percent sperm with active mitochondria and low PLA activity were increased in dose dependent manner ($P<0.05$).

Introduction

To achieve optimum output from livestock, artificial insemination (AI) with frozen semen is a major breeding tool. Artificial insemination with cryopreserved semen is a widely used technique in buffalo (Singh and Balhara, 2016). However, acceptable conception rates not achieved under field conditions with frozen-thawed buffalo semen remained a challenge so far. Also, the fertility of cryopreserved semen remains poor (33%) as compared to fresh semen (Chohan *et al.*, 1992). This might be due to lower freezability and fertility of buffalo spermatozoa when compared with cattle spermatozoa (Singh and Pant, 2000). One important reason for poor fertility of cryopreserved semen is freezing induced apoptosis like changes inflicted in spermatozoa during cryopreservation indicated by externalization of phosphatidylserine (PS) due to higher

phospholipase activity (Glander *et al.,* 2002). Caspases are synthesized as non-active proenzyme (pro-caspases) which are activated by cleavage during the cascade of ordered events of apoptosis (Cohen, 1997). Apoptosis-like changes has been identified by the presence of caspase 9 and caspase 3 in bovine sperm (Anzar *et al.,* 2002), whereas increased membrane permeability and decreased mitochondrial membrane potential has been observed in equine sperm (Ferrusola *et al.,* 2008).

Martin *et al.,* (2004) also found that, after cryopreservation, majority of living sperm cells show low mitochondrial potential. The caspases activate DNase and are responsible for DNA fragmentation (Enari *et al.,* 1998) and as a result, 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). The existence of caspase-dependent apoptotic-like mechanisms associated with mitochondrial functionality in sperm, are possibly similar to those found in somatic cells (Boise and Thompson, 1997; Ricci *et al.,* 2003, 2004; Lakhani *et al.,* 2006). Dalal *et al.,* (2018) reported that Z-DEVD-FMK improves post thaw percent sperm with active mitochondria and low PLA activity increases in dose dependent manner.

They also found that there was improvement in post thaw sperm viability with intact membrane following Z-DEVD-FMK (caspase inhibitor) treatment (2, 4 and 6 µM). Z-LEHD-FMK inhibits caspase 9 (Alicia *et al.,* 2006) and Caspase 9 triggers a cascade of caspase activation, including caspase-3/7 that promotes cellular self-destruction (Paasch *et al.,* 2004; Bejarano *et al.,* 2008). So, the improvement in quality of post thaw semen could be achieved by minimizing apoptosis like changes developed during cryopreservation.

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 of Murrah buffalo bulls.

Selection of buffalo bulls

Three breeding buffalo bull around 4 years of age maintained at bull farm, GADVASU, Punjab, India (Latitude/Longitude, 30.55°N, 75.54° E) were included in the present study. These bulls were used for routine 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

Four ejaculates, each from 3 buffalo bulls were taken in this study. Tris egg yolk extender was prepared as described by Dalal *et al.,* (2017). Caspase inhibitor was dissolved in dimethylsulphoxide (DMSO) to prepare the stock solution (10 mM). Z-LEHD-FMK (0, 2 µM, 4 µM, 6 µM, 10 µM and 20 µM) was mixed into the semen extender to suppress the apoptosis like changes during cryopreservation. Six aliquots from each ejaculate were extended (80 millions sperm/ml) with tris egg yolk extender to corresponding Z-LEHD-FMK concentrations and were frozen using standard vapour freezing method. Sperm samples were analyzed for individual motility, viability, plasma membrane integrity, mitochondrial membrane potential and status of sperm membrane phosphatidylserine through PLA

activity at pre-freeze and post thaw stages was assessed. The individual sperm motility (%) was assessed manually under 20 X objective of phase contrast microscope (Nikon Eclipse E 200). The live sperm count (%) was performed using Eosin-Nigrosine staining technique as per standard procedure (Blom *et al.,* 1977). The hypo-osmotic swelling test (HOST) was performed as per standard procedure to assess the functional integrity of sperm membrane (Jeyendran *et al.,* 1984; Dalal *et al.,* 2016).

Evaluation of mitochondrial membrane potential

Mitochondrial membrane potential was assessed by using fluorescent dye Tetra-methyl rhodamine, methyl ester (TMRM, Life Technologies; Cat#T-668) as described by Dalal *et al.,* (2016, 2018). Briefly, stock solution (10mM) of TMRM was prepared. From stock solution of TMRM, a working solution was prepared (50 µM) and stored at -20^{o}C.After two washing with PBS at 1000 rpm for 5 min, 5µl of TMRM was added to each sperm sample and incubated at 37^{0}C for 90 min. After incubation, washing was done with PBS for 5 min at 37^{0}C to remove 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), fluorescein isothiocyanate filter (FITC) (510 - 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

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, 20 µM of working solution prepared from stock (100mM in DMSO).

Following two washing with PBS at 1000 rpm, 30 µl of BODIPY working solution was mixed to each sperm sample and incubated for 45 min at 37 ^{0}C. After incubation, washing was done with 1ml of PBS at 1000 RPM for 5 minutes at 37^{0}C to remove 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 AntifadeMountant 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). Around 100 sperms in different fields were observed and out of hundred, normal sperms without fluorescence were calculated as % sperm with low PLA (phospholipase A1 and A2) activity.

Statistical analysis

All statistical analyses were carried at 0.05 probability level out using IBM SPSS Statistics software (IBM Corporation, USA) for windows. Data were analyzed using one-way ANOVA and comparison of means was done by Duncan Multiple Range Test (DMRT). Results are expressed as Mean±Standard Error of Mean. Statistical significance was set at 0.05 probability level.

Results and Discussion

In this study, Z-LEHD-FMK (0, 2, 4, 6, 10, and 20 µM) was used in tris egg yolk extender to reduce the apoptosis like changes in sperms. Evaluation of the pre-freeze and post-thaw semen samples parameters like percent sperm motility, viability, plasma membrane integrity,

mitochondrial membrane potential, and sperm PLA activity status is shown in Table 1.

Sperm Motility

There was no effect of Z-LEHD-FMK (P>0.05) on percent motility before freezing. However, percent post thaw motility in 2µM (55.45±3.78) concentration of Z-LEHD-FMK was higher (P<0.05) than other treatments (4, 6, 10 and 20 µM) and control (45.54±3.34). The mechanisms of inducing apoptosis by different caspases are more complex and many factors may be involved (Sule *et al.,* 2013). However, in a study Dalal *et al.,* (2018) did not find any significant (P>0.05) effect on percent motility following Z-DEVD-FMK treatment. Furthermore, Chen *et al.,* (2006) reported an inverse relationship between sperm motility and apoptosis in human sperms.

Sperm viability

In the present study, the percent viable sperms count was similar (P>0.05) in pre-freeze sperms both in treated and control group. However, the percent viable sperms count at post thaw stage was higher (P < 0.05) in 2 µM concentration (86.36±3.67) of Z-LEHD-FMK as compared to other 4 (66.66±6.32), 6 (76.78±5.78), 10 (52.46±7.31) and 20 µM (48.57±5.65) and control (48.78±7.34).

Percent viable sperms count in post-thaw samples was similar (P>0.05) between Z-LEHD-FMK treated (10, 20 µM) and control samples. But these showed lesser viable sperm count as compared to 2, 4 and 6 µM treatments. In a previous study, Z-DEVD-FMK (2 and 4 µM) treatment also improved (P<0.05) the sperm viability (Dalal *et al.,* 2018). These caspase inhibitors inhibited the caspases that share some of the apoptosis pathways and more or less oppose the apoptosis.

Hypo osmotic swelling test (HOST)

Before freezing, there were no difference (P>0.05) in percent sperms with intact plasma membrane between control and Z-LEHD-FMK (2, 4, 6, 10 and 20 µM) as assessed through HOST. Host reactive sperms in post thaw samples were higher (P<0.05) in 2 µM (66.64±4.89) as compared to other concentration of Z-LEHD-FMK like 4 µM (52.79±4.99), 6 µM (56.87±5.34), 10 µM (44.56±5.33) 20 µM (50.31±6.66) and control (51.5±3.73).

So, Z-LEHD-FMK protects the plasma membrane in lower concentrations. In previous study (Dalal *et al.,* 2018), Z-DEVD-FMK treatment also reported to protect the plasma membrane (P<0.05).

Mitochondrial status

The percent active mitochondria were similar (P > 0.05) in Z-LEHD-FMK ((2, 4, 6, 10 and 20 µM) treated and control before freezing. However, in post thaw sperm, percent active mitochondria were higher (P < 0.05) in 6 µM (75.56±3.45), 10 µM (78.11±2.27) and 20 µM (82.40±2.56) concentrations of Z-LEHD-FMK as compared to control (65.6 ± 3.45).

The percent active mitochondria in post thaw samples did not differ (P>0.05) in2 µM (67.12±4.12), 4 µM (70.34±4.44) and control (60.66±3.89).

Also, percent active mitochondria in 20 µM were higher (P<0.05) as compared to and 10 µM (77.5 ± 1.45) doses. Previously, Dalal *et al.,* (2018) also recorded improvement in maintenance of mitochondrial potential with Z-DEVD-FMK following cryopreservation. Hence, Z-LEHD-FMK help in maintaining mitochondrial membrane potential of spermatozoa in dose dependent manner following cryopreservation of semen.

Table.1 Effects of different treatments of Z-LEHD-FMK on various sperm parameters 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 (%)	80.28±8.77	85.67±7.89	85.23±6.99	85.59±5.55	85.29±5.81	80.67±7.45	45.54±3.34^{a}	55.45±3.78^{b}	45.97±4.65^{a}	45.56±4.67^{a}	50.34±4.61^{ab}	47.66±4.56^{a}
Live sperm (%)	80.67±5.22	82.0±3.56	74.45±8.61	78.67±8.32	80.87±6.55	74.62±4.98	48.78±7.34^{a}	86.36±3.67^{c}	66.66±6.32^{b}	76.78±5.78^{bc}	52.46±7.31^{a}	48.57±5.65^{a}
Sperm with intact PM(%)	74.56±5.88	78.48±6.45	66.19±5.47	68.52±6.88	64.45±5.87	68.44±3.67	51.5±3.73^{a}	66.64±4.89^{b}	52.79±4.99^{a}	56.87±5.34^{a}	44.56±5.33^{a}	50.31±6.66^{a}
Active MT* (%)	78.11±2.35	80.67±2.89	82.66±5.31	83.53±3.71	85.45±4.66	84.6±2.56	60.66±3.89^{a}	67.12±4.12^{ab}	70.34±4.44^{ab}	75.56±3.45^{b}	78.11±2.27^{bc}	82.40±2.56^{c}
Sperm with low PLA activity (%)	75.89±3.67	75.21±4.58	83.34±3.37	84.21±3.78	82.67±5.12	81.5±2.66	60.23±3.23^{a}	70.34±3.36^{b}	74.55±2.48^{b}	73.76±3.12^{b}	78.87±4.23^{bc}	82.55±4.11^{c}

Values marked with dissimilar superscript differ significantly ($P < 0.05$) within a row. PM and MT refers to plasma membrane and mitochondria of sperms, respectively.

PLA activity

Before freezing, there were no difference ($P < 0.05$) between control and Z-LEHD-FMK (2, 4, 6, 10 and 20 µM) treated samples in terms of percent sperms with low PLA activity. However, the percent sperms with low PLA activity in post thaw samples were higher ($P < 0.05$) in 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) concentrations of Z-LEHD-FMK as compared to control (60.87 ± 4.68). The percent sperms with low PLA activity in post thaw samples were higher ($P < 0.05$) in 10 and 20 µM of Z-LEHD-FMK as compared to lower concentrations. Our study indicated that Z-LEHD-FMK supplementation has protective effect against apoptosis like changes in spermatozoa during cryopreservation as reported previously by Dalal *et al.*, (2018) with the use of Z-DEVD-FMK.

To the best of our knowledge, this is the first report on use of Z-LEHD-FMK to minimize apoptosis like changes in buffalo sperm induced during cryopreservation. Cryopreservation induce increase in caspase activation in human sperm positive for active 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). The increase in caspase activation is dependent on the sperm preparation and cryopreservation protocol (Grunewald *et al.*, 2005). Cryopreservation has been reported to activate caspase-3 and -9 in humans (Paasch *et al.*, 2004; Bejarano *et al.*, 2008) and in boar

sperms (Van Gurp *et al.,* 2003). Caspase activation following the cryopreservation and thawing process is also reported in cattle (Martin *et al.,* 2004; 2007) and equine spermatozoa (Brum *et al.,* 2008; Ferrusola *et al.,* 2008). It is well known fact that during apoptosis, mitochondrial pores open causing releasing of pre-apoptotic proteins like CytochromeC (an electron transport chain component) from inner membranous space to outside which activates caspase 9 in bovines (Reed 1997a). Bejarano *et al.,* (2008) reported that Z-LEHD-FMK abolished H_2O_2-induced phosphatidylserine exposure. Similarly, they also found that progesterone induced PS externalization was dependent on caspase-9 activation. However, progesterone present in substantial amounts in egg yolk is being routinely used to prepare semen extenders. Bertin *et al.,* (2013) reported that progesterone concentrations in egg yolk as 1823.71 ± 64.24 ng/g. As 20 %egg yolk is used in semen extender, so progesterone of egg yolk in semen extender might induced PS externalization.

Also, Cytochrome C released due to mitochondrial damage does not activate procaspase-8, but, is known to primarily activate procaspase-9 (Peter *et al.,* 2003). Caspase 9 triggers a cascade of caspase activation, including caspase-3/7 that promotes cellular self-destruction (Paasch *et al.,* 2004; Bejarano *et al.,* 2008). Caspase 3 and 7 are the executioner proteome of apoptosis (Vilmont *et al.,* 2012). It has been reported that Z-LEHD-FMK selectively inhibits the caspase 9 (Alicia *et al.,* 2006).

Procaspase-9 was reported in cattle bull sperm, but procaspase-3 and -8 were absent and cryopreservation had been reported to activate caspase-9 in cattle bull sperm (Martin *et al.,* 2007). Our study indicates that lower concentration (2 µM) of Z-LEHD-FMK improves the sperm motility, viability and sperm membrane integrity whereas percent sperm count with high mitochondrial potential and low PLA activity of sperm were increased in dose dependent manner. Furthermore, additional depth studies will be required to assess the other properties of caspase inhibitors. 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 of ram (Marti *et al.,* 2008), dog(Peter and Linde-Forsberg, 2003) and stallion sperms (Peter *et al.,* 2005). These differences from our study may be due to the species variationor differences in doses of supplementation. Peter *et al.,* (2005) also suggested that a higher or lower level of caspase doses with different timings of treatment may produce the desired effects.

In conclusion, the Z-LEHD-FMK improves spermmotility, viability and plasma membrane integrity in lower concentrations together with maintenance of mitochondrial potential with low PLA activity in post thaw semen samples in a dose dependent manner. It implies that apoptosis like changes developed during cryopreservation and Z-LEHD-FMK helps to counteract these apoptosis-like changes in sperms.

Conflict of interest statement

The authors declare no conflict of interests (financial or nonfinancial) with any organization or entity.

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