Original Research Article

Effect of Z-IETD-Fmk (Caspase Inhibitor) Supplementation on Apoptosis Like Changes Developed in Buffalo Bull Sperm during Cryopreservation

Jasmer Dalal[1*], AjeetKumar[2], M. Honparkhe[1] and P.S. Brar[1]

[1]Department of Veterinary Gynaecology and Obstetrics, Lala Lajpat University of Veterinary and Animal University, Hisar, 125004, India
[2]Department of Veterinary Gynaecology and Obstetrics, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana 141 004, Punjab, India

Corresponding author

A B S T R A C T

Present study was conducted to evaluate the anti-apoptotic effect of caspase inhibitor (Z-IETD-FMK) supplementation in buffalo bull semen. The Z-IETD-FMK was supplemented with Tris egg yolk extender @ 2, 4, 6, 10 and 20 µM. The 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 (P>0.05) of Z-IETD-FMK treatment on sperm motility (%), sperm viability (%) and percent sperm with active mitochondria at pre-freeze and post-thaw stages. However, there was improvement in terms of % HOS reactive sperms and % sperms with low PLA activity in higher supplementation doses (20 µM) of Z-IETD-FMK in post freeze semen samples as compared to control. Thus Z-IETD-FMK shows anti-apoptotic effect in higher doses.

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 activate DNase 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, Ricci *et al.,* 2004: Lakhani *et al.,* 2006).

The Z-IETD-FMK inhibits caspase 8 (Alicia *et al.,* 2006). The caspase-8 has been detected in refrigerated ram sperm samples (Mendoza *et al.,* 2013). Extrinsic and intrinsic are two main pathways of apoptosis. Former is initiated by binding of extracellular death ligand like ExoS ligand (FasL) to cellular death receptor like Fas (Ashkenazi and Dixit 1998), later is mediated by mitochondrial alterations. Caspase-8 is a key connecting link between two propagation pathways of apoptosis, especially when stimulated by external cytokines (Lee *et al.,* 1999). So, supplementation of caspase 8 inhibitor, Z-IETD -FMK could be of use in minimizing apoptosis like changes (Alicia *et al.,* 2006).

Materials and Methods

Ethical approval

As the present did not involve handling of live animals and no invasive technique was used so, approval from the institutional animal ethics committee was not required and semen was being collected and frozen as a routine procedure under progeny testing program.

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) 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-IETD - 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- IETD-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; Dalal *et al.,* 2016).

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 described previously (Dalal *et al.,* 2016; Dalal *et al.,* 2018; Dalal *et al.,* 2018a; Dalal *et al.,* 2018b). 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, 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 described previously (Dalal *et al.,* 2016; Dalal *et al.,* 2018; Dalal *et al.,* 2018a; Dalal *et al.,* 2018b). 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-IETD-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 Z-IETD-FMK treated and control groups as shown in Table 1. Similarly, post thaw % motility in IETD-FMK treated and control groups 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-IETD -FMK did not affect % sperm motility. Chen *et al.,* (2006) reported the inverse relationship between sperm motility and apoptosis in human spermatozoa. Previously, also it has been reported that sperm motility was similar when treated with different concentration of Z-DEVD-FMK (Dalal *et al.,* 2018a) and Z-LEHD -FMK (Dalal *et al.,* 2018b).

Sperm viability

There was no significant (P > 0.05) difference in % sperm viability in pre-freeze IETD-FMK treated and control groups (Table 1). Similarly, percent viable sperms in post thaw samples were similar (P > 0.05) between Z-IETD -FMK treated and control groups. However, in previous studies sperm viability was improved (P<0.05) in lower doses of Z-DEVD-FMK (Dalal *et al.,* 2018a) and Z-LEHD -FMK (Dalal *et al.,* 2018b).

Hypo osmotic swelling test (HOST)

In pre-freeze samples, there were no significant (P < 0.05) difference between control and Z-IETD -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 @ 20 μM (71.19±5.89) than control (59.33±5.22). The % Host reactive sperms were similar in post thaw samples significantly among 2 (62.64±5.09), 4 (65.12±6.83), 6 (63.18±7.44) and 10μM (65.29±5.61) and did not differ significantly from control and 20 μM treated group. In previous study, Dalal *et al.,* (2018b) demonstrated that 2μM of Z-DEVD -FMK protect the functional integrity of sperm plasma membrane. In another study with

other caspase inhibitor, Dalal *et al.,* (2018a) reported that Z-LEHD-FMK in lower dose (2 and 4μM) protects the functional integrity of buffalo sperm.

Mitochondrial status

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

In post thaw samples, the % active mitochondria were also similar (P> 0.05) in Z-IETD -FMK supplemented and control groups. Hence, supplementation of Z-IETD-FMK did not affect mitochondrial membrane potential of spermatozoa following cryopreservation of semen. However, in previous studies Z-DEVD-FMK (Dalal *et al.,* 2018a) and Z-LEHD -FMK (Dalal *et al.,* 2018b) improved the mitochondrial potential.

PLA activity

In pre-freeze semen samples, there were no significant (P < 0.05) difference between control and Z-IETD-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 @ 20 μM (76.56±5.23) of Z-IETD-FMK than control (61.56±5.98). The % sperms with low PLA activity were similar in post thaw samples significantly among 2 (63.44±6.37), 4 (65.11±5.88), 6 (69.71±8.55) and 10μM (73.19±6.28) of Z-IETD-FMK and did not differ significantly from control and 20 μM treated group.

Our study indicated that Z-IETD-FMK supplementation has protective effect against

apoptosis like changes in spermatozoa during cryopreservation especially in higher doses (20 µM) as reported previously with Z-DEVD-FMK(Dalal *et al.*, 2018a) and Z-LEHD-FMK (Dalal *et al.*, 2018b) but in these study effect was also observed in lower doses indicating that Z-DEVD-FMK and Z-LEHD-FMK inhibitors are more potent than Z-IETD-FMK in protecting the sperm from cryopreserved induced apoptosis.

Table.1 Effects of supplementation of Z-IETD-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	91.12± 6.23[a]	85.23± 5.48[a]	85.51± 6.24[a]	90.76± 7.11[a]	86.34± 5.89[a]	90.34± 6.75[a]	50.55± 5.38[a]	55.57± 7.79[a]	50.43± 8.34[a]	52.46± 5.45[a]	45.23± 6.88[a]	45.29± 5.78[a]
% Viability	90.56± 4.33[a]	95.17± 5.88[a]	89.81± 7.73[a]	88.62± 6.89[a]	80.56± 7.49[a]	85.45± 5.85[a]	60.22± 6.47[a]	65.48± 6.31[a]	55.87± 7.39[a]	58.11± 5.29[a]	60.81± 6.39[a]	67.73± 4.59[a]
% HOS reactive sperm	82.56± 3.45[a]	83.46± 5.76[a]	81.37± 7.23[a]	85.68± 5.91[a]	88.14± 4.38[a]	85.18± 3.84[a]	59.33± 5.22[a]	62.64± 5.09[a]	65.12± 6.83[ab]	63.18± 7.44[ab]	65.29± 5.61[ab]	71.19± 5.89[b]
% Active mitochondria	81.78± 7.99[a]	83.45± 3.49[a]	75.55± 4.64[a]	78.42± 5.32[a]	75.12± 3.56[a]	78.68± 4.88[a]	55.52± 5.87[a]	60.71± 4.99[a]	52.53± 6.85[a]	57.48± 4.68[a]	50.83± 5.83[a]	57.44± 6.24[a]
% sperms with low PLA activity	78.34± 3.48[a]	75.31± 5.77[a]	81.45± 4.37[a]	85.28± 6.27[a]	86.45± 5.37[a]	88.11± 4.77[a]	61.56± 5.98[a]	63.44± 6.37[ab]	65.11± 5.88[ab]	69.71± 8.55[ab]	73.19± 6.28[ab]	76.56± 5.23[b]

To the best of our knowledge, this is the first report on use of Z-IETD-FMKto 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) and each species has its own optimum freezing rates

(Dalal *et al.,* 2018). 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;Martin *et al.,*2007) and equine spermatozoa (Brum *et al.,* 2008; Ferrusola *et al.,* 2008).

The Z-IETD-FMK is a powerful cell permeable selective inhibitor of caspase 8 (Alicia *et al.*, 2006). Caspase 8 is involved in the membranous pathway of apoptosis. After stimulation by external death signals, caspase-

8 can directly either activate executioner caspases or convert BID (BH3 interacting domain) into tBid (truncated BID), which translocate from cytosol to mitochondria where it enhances permeability and release of cytochrome *c* (Lee *et al.,* 1999). Thus, Caspases 8 act through membranous pathway of apoptosis (Slee *et al.,* 1999) and membranous pathway is less commonly operated in bovine sperm (Zou *et al.,* 1999). However, Z-IETD-FMK partially inhibits the caspase 3 as reported treatment of retinal cells with 20μM of the selective inhibitor of caspase-8, Z-IETD-FMK, resulted in decreased caspase-3like activity only in retinal cells (Gülgün and Martin, 1999). In present study also, Z-IETD-FMK showed antiapoptotic effect but in higher concentration (20μM) suggesting that in lower concentration it partially inhibit caspase 3. However higher concentration of Z-IETD-FMK might cause more potent inhibitory effect on caspase 3.

Our study indicates that higher concentration (20 μM) of Z-IETD –FMK protect the sperm membrane integrity and prevented externalization of phosphatidylserinesperm with low PLA activity of sperm based on the fact that spermatozoa with deteriorated membrane and externalized phosphatidylserine are characterized by an increased lyso-phosphatidylcholine content that is likely generated by phospholipases (Glander *et al.,* 2002). Furthermore, additional depth studies will be required to assess the other properties of caspase inhibitors to revealed exact mechanisms of actions. 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 variation

or 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-IETD -FMK improves spermplasma membrane integrity in higher concentrations together with maintenance of low PLA activity in post thaw sperm. It implies that apoptosis like changes developed during cryopreservation and Z-IETD -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.

Acknowledgments

References

Alicia, B. B., Sexton, K. B. and Bogyo, M. (2006). Commonly used caspase inhibitors designed based on substrate specificity profiles lack selectivity. *Cell research*. 16: 961-63.

Anzar, M., Liwei, He., Mary, M., Buhr, T.G. and Kroetsch, P. K.P. (2002). Sperm apoptosis in fresh and cryopreserved bull semen detected by flow cytometry and its relationship with fertility. *Biol. Reprod.,* 66: 354-60.

Ashkenazi, A. and Dixit, V.M. (1998). Death receptors: signaling and modulation.

Science 281: 1305-08.

Bejarano, I., Lozano, G.M., Ortiz, A., Garcia, J.F., Paredes, S.D., Rodrı´guez, A.B. and Pariente, J.A. (2008). Caspase-3 activation in human spermatozoa in response to hydrogen peroxide and progesterone. *Fertil. Steril.,* 90: 1340–1347.

Blom, E. (1977). The evaluation of bull semen with special reference to its use in artificial insemination. *Anim. Breed. Abstr*., 19: 648.

Boise, L.H. and Thompson, C.B. (1997). Bcl-x(L) can inhibit apoptosis in cells that have undergone Fas-induced protease activation. *Proc. Natl. Acad. Sci*., USA; 94:3759–3764.

Brum, A.M, Sabeur, K. and Ball, B.A. (2008). Apoptotic-like changes in equine spermatozoa separated by density-gradient centrifugation or after cryopreservation. *Theriogenology*. 69: 1041–1055.

Chen, Z., Hauser, R., Trbovich A M, Shifren J L, Dorer D J, Bailey L G and N P Singh. (2006). The Relationship between Human Semen Characteristics and Sperm Apoptosis: A Pilot Study. *Journal of Andrology,* 27: 112-20.

Chohan, K.R., Iqbal, J., Asghar, A.A. and Chaudhry, M.A. (1992). Fertility of liquid and frozen semen in Nili Ravi buffaloes. *Pak. Vet. J.,* 12:4–5.

Cohen, G. M. (1997). Caspases: the executioners of apoptosis. *Biochem. J*., 326: 1-16.

Dalal, J., Kumar, A., Dutt, R., Singh, G. and Chandolia, R.K. (2018). Different Cooling Rates for Cryopreservation of Semen in Various Livestock Species: A Review. *Int.J.Curr.Microbiol. App.Sci.* 7(08): 1903-1911

Dalal, J., Kumar, A., Honparkhe, M., Deka, D. and Singh, N. (2016). Minimization of apoptosis-like changes in cryopreserved buffalo bull sperm by supplementing extender with Bcl-2 protein. *Vet. World.,* 9(5): 432-436.

Dalal, J., Kumar, A., Honparkhe, M., Singh., Singh A.K. and Brar, P.S. (2018a). Minimization of Apoptosis like Changes by Supplementing Z-DEVD-FMK (CASPASE inhibitor) during Cryopreservation of Buffalo Bull Semen. *Int.J.Curr.Microbiol.App.Sci.,* 7(07): 3333-3340.

Dalal, J., Kumar, A., Honparkhe, M., Singh., Singh A.K. and Brar, P.S. (2018b). Minimization of Apoptosis like Changes Developed during Cryopreservation of Buffalo Bull Sperm by Supplementing Z-LEHD-FMK (Caspase Inhibitor). *Int. J. Curr. Microbiol.App.Sci.* 7(09): 1076-1084.

Dalal, J., Kumar, A., Honparkhe, M., Singhal, S. and Singh, N. (2016). Comparison of three programmable freezing protocols for the cryopreservation of buffalo bull semen. *Ind. J. Ani. Reprod.,* 37 (2):54-55.

Dalal, J., Kumar, A., Kumar, P., Honparkhe, M., Malik, V.S., Singhal, S., Kaur, S. and Brar, P.S. (2018). Improvement in cryosurvival of buffalo bull (*Bubalus bubalis*) sperm by altering freezing rate within critical temperature range. *Asian. Pac. J. Reprod.,* 7(2): 72-78.

Danial, N.N. and Korsmeyer S J. (2004). Cell death: critical control points.*Cell*.11: 205-19.

Enari. M., Sakahira, H., Yokoyama, H., Okawa, K., Iwamatsu, and Nagata, S. (1998). A caspase activated DNase that degrades DNA during apoptosis, and its inhibitor ICAD. *Nature*. 391: 43-50.

Erickson, L., Kroetsch, T. and Anzar, M. (2015). Relationship between sperm apoptosis and bull fertility: in vivo and in vitro studies. *Reprod. Fertil. Dev*., 28(9): 1369-1375. doi:

10.1071/RD14417

Ferrusola, C.O., Galan, Y.S., Fernandez, E.V., Bolanos, J.M.G., Muriel, A., Fernandez, L.G., Tapia, J.A. and Pena, F.J. (2008). Detection of ''apoptosis-like'' Changes during the cryopreservation process in equine sperm. *J. Androl*., 29: 213-21.

Glander, H.J., Schiller, J., Süss, R., Paasch, U., Grunewald, S. andArnhold, J. (2002). Deterioration of spermatozoal plasma membrane is associated with an increase of sperm lyso-phosphatidylcholines.*Andrologia*. 34(6): 360-6.

Grunewald, S., Paasch, U., Wuendrich, K. andGlander, H.J. (2005). Sperm caspases become more activated in infertility patients than in healthy donors during cryopreservation. *Arch. Androl.,* 51:449–460.

Gülgün, T. and Martin B.W. (1999). Inhibition of caspase activity in retinal cell apoptosis induced by various stimuli *in vitro*. *Invest. Ophthalmol. Vis. Sci*., 40 (11):2660-2667.

Jeyendran, R.S., Vander-Ven, H.H., Perez-Pelaez, M., Crabo, B.G. andZanevld, L.J.D. (1984). Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characters. *J. Reprod. Fertil*., 70: 219-28.

Lee, J., Richburg, J.H, Shipp, E.B, Meistrich, M.L and Boekelheide K. (1999). The Fas system, a regulator of testicular germ cell apoptosis, is differentially up-regulated in Sertoli cell versus germ cell injury of the testis. *Endocrinology*140: 852-58.

Marti, E., Perez-Pe R. and Colas, C. (2008). Study of apoptosis-related markers in ram spermatozoa. *Anim. Reprod. Sci*., 106: 113–132.

Martin, G., Cagnon, N. and Sabido, O. (2007). Kinetics of occurrence of some features of apoptosis during the cryopreservation process of bovine spermatozoa. *Hum. Reprod*., 22: 380-88.

Martin, G., Sabido, O., Durand, P. and Levy1, R. (2004). Cryopreservation induces an apoptosis-like mechanism in bull sperm. *biol. Reprod*., 71:28-37.

Mendoza, N., Casao, A., Pérez-Pé, R., Cebrián-Pérez, J.A., Muiño-Blanco T. (2013). New Insights into the Mechanisms of Ram Sperm Protection by Seminal Plasma Proteins. *Biol. Reprod.,*88(6): 149, 1–15.

Paasch, U., Sharma, R.K., Gupta, A.K., Grunewald, S., Mascha, E.J., Thomas. A.J., Glander, J.H. J. and Agarwal, A. (2004). Cryopreservation and thawing is associated with varying extent of activation of apoptotic machinery in subsets of ejaculated human spermatozoa. *Biol. Reprod*., 71:1828-37.

Peter, A.T. and Linde-Forsberg, C. (2003). Efficacy of the anticaspase agent zVAD-fmk on post-thaw viability of canine spermatozoa. *Theriogenology.* 59:1525–1532.

Peter, A.T., Colenbrander, B. andGadella, B.M. (2005). Effect of caspase inhibitors on the post-thaw motility, and integrity of acrosome and plasma membrane of cryopreserved equine spermatozoa. *Indian. J. Exp. Biol.,*43:483–487.

Ricci, J.E., Gottlieb, R.A. and Green, D.R. (2003). Caspase-mediated loss of mitochondrial function and generation of reactive oxygen species during apoptosis. *J. Cell. Biol*., 160: 65–75.

Ricci, J.E., Munoz-Pinedo, C., Fitzgerald, P., Bailly-Maitre, B., Perkins, G.A., Yadava, N., Scheffler, I.E., Ellisman, M.H. and Green, D.R. (2004). Disruption of mitochondrial function during apoptosis is mediated by

caspase cleavage of the p75 subunit of complex I of the electron transport chain. *Cell.* 117:773–786.

Singh, I. and Balhara, A.K. (2016).New approaches in buffalo artificial insemination programs with special reference to India. *Theriogenol.*, 86(1): 194-199.

Slee, E.A, Harte, M.T., Kluck, R.M, Wolf, B.B, Casiano, C.A., Newmeyer, D.D, Wang, H.G, Reed, J.C, Nicholson, D.W and Alnemri, E.S. (1999). Ordering the cytochrome c-initiated caspase cascade: hierarchical activation of caspases-2, -3, -6, -7, -8, and -10 in a caspase-9-dependent manner. *Journal of Cell Biology*144: 281-92.

Sule, D., Mason, M.C., Aruna, G., Lauren, B., Abdullah, K., John, S., Dennis, R. and Erdogan, M. (2013). Interrelationships between apoptosis and fertility in bull sperm. *J. Reprod. Dev.,* 59: 18-26.

vanGurp, M., Festjens, N., van Loo, G., Saelens, X. and Vandenabeele, P. (2003). Mitochondrial intermembrane proteins in cell death. *Biochem. Biophys. Res. Commun.*, 304: 487–97.

Zou, H., Yuchen, L., Xuesong, L. and Xiaodong, W. (1999). An APAF-1zCytochrome *c* Multimeric Complex Is a Functional Apoptosome That Activates Procaspase-9. *J. Biol. Chem.,* 274(17): 11549-56.