IJAS Iranian Journal of Applied Animal Science

Fortification of Catalase Improves Post Thaw Fertility of Goat Semen

Research Article

R. Ranjan[1*], P. Singh[1], C. Gangwar[1], S.P. Singh[1], D.K. Swain[2] and S.D. Kharche[1]

[1] Department of Animal Physiology and Reproduction, Institute of Indian Council of Agricultural Research (ICAR)-Central Institute for Research on Goats, Farah, Mathura, Uttar Pradesh, India
[2] Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go-Anusandhan Sansthan (DUVASU), Mathura, Uttar Pradesh, India

Received on: 7 Jun 2020
Revised on: 14 Sep 2020
Accepted on: 15 Oct 2020
Online Published on: Sep 2021

*Correspondence E-mail: ravi.ranjan@icar.gov.in

Online version is available on: www.ijas.ir

ABSTRACT

Artificial Insemination (AI) in goats is less developed compared to the large animals due to the lack of suitable protocol of goat semen freezing and AI in India. The AI plays a pivotal role in the long-term ex-situ *in vitro* conservation of threatened breeds, increased productivity, and performance of a large number of non-descript and low potential goats. The objective of the present study was to enhance the life and fertility potential of cryopreserved semen and consequently the conception rate through frozen semen AI by the addition of catalase in an extender. Ejaculates (30) were collected and were extended with tris-citric acid-fructose diluent. Catalases were added in diluent (0 IU/mL, 200 IU/mL, 400 IU/mL, 600 IU/mL, 800 IU/mL and 1000 IU/mL). Analysis of data using SPSS 16 revealed that motility, live sperm count, acrosomal integrity, and hypo-osmotic swelling positive spermatozoa were counted differed significantly ($P<0.05$) at different concentrations of catalase. The post-thaw motility, live sperm count, acrosomal integrity, and hypo-osmotic swelling positive spermatozoa were significantly ($P<0.05$) highest in 800 U/mL of catalase used in the present study. Mitochondrial membrane potential was also significantly highest ($P<0.05$) in frozen semen in 800 IU/mL catalase. Goats (20) were inseminated with frozen semen straw of 800 IU/mL catalase and 8 goats (40%) were pregnant. The conception rate in the control group was 35%. Our study suggested that the addition of catalase reduced the detrimental effects of freezing on motility, viability, plasma membrane and acrosome integrity and can be used for routine semen freezing and AI.

KEY WORDS antioxidant, catalase, goat, mitochondrial membrane potential, post thaw semen.

INTRODUCTION

India has 148.88 million goats with 38 recognized goat breeds that are well adapted to different agro-climatic regions of this country. The unique characteristic of the goat is to produce on minimum available feed resources under extreme environmental conditions which make it suitable for domestication (Gama and Bressan, 2011). There is a scarcity of quality breeding bucks due to the early castration and slaughter of male goats for meat purpose. The descript goat population (33%) is very less compared to non-descript and non-productive goats (67%) (Acharya, 1987).

We need 2 million bucks to cover 85 million breedable does for natural breeding as compared to only 50000 bucks needed for frozen semen AI technology in India. AI is an effective tool in crossbreeding programs of non-descript indigenous goats with superior germplasm to enhance productivity in India (Leboeuf *et al.* 2000; Rahman *et al.* 2008). In our country goats are mostly reared by small, marginal and landless farmers, with a flock size of 3-5 goats so it is economically unviable to keep a breeding buck for a small flock (Acharya, 1987). Thus, the females are bred by nondescript males resulting in the loss of precious germplasm of this region. Therefore, the preservation

of superior germplasm is important to sustain the unique qualities of the breed and to prevent the dilution of valuable germplasm by indiscriminate mating. This can be achieved by adopting AI which allows faster transmission of genetic merit. The practice of AI is gaining momentum in goat farming sector for genetic upgradation of local non-descript goat population to fulfill the hunger and health needs of the increasing human population.

Cooling of spermatozoa depresses spermatozoal metabolic rate to prolong sperm survival. Cooled semen has low motility and morphological integrity and declined survival ability in the female reproductive tract compared to fresh semen. Reactive oxygen species (ROS) is the main culprit behind these damages that leads to oxidative stress further resulting in increased rates of lipid peroxidation and consequent loss of sperm motility during long-term storage (Aitken, 2017). Various additives have been added to extenders to maintain motility and fertilizing capacity and to preserve the integrity of the sperm membrane (Saraswat *et al.* 2012; Ranjan *et al.* 2017). Mostly, these protectants possess antioxidant activity and either reduce the process of oxidation (Pietta, 2000) or regulate, suppress or prevent the formation of ROS (Maneesh and Jayalekshmi, 2006). The addition of catalase as an antioxidant into semen extenders improved semen quality in ram (Gungor *et al.* 2018), bovine (Bilodeau *et al.* 2002), equine (Baumber *et al.* 2002). However, few reports are available on the effect of ROS on goat sperm in addition to cryoinjuries encounters lethal interactive losses.

Freezing and thawing of buck semen cause changes in sperm motility, morphology including damage to mitochondria, plasma membrane, and acrosome intactness thereby, reducing the fertilizing ability of spermatozoa. Freezing and thawing resulted in structural change in plasma membrane permeability and damage of mitochondria (Kadirvel *et al.* 2012). The mitochondrial membrane potential (MMP), responsible for ATP production, is indispensable for the flagellar beat and sperm motility (Flesch and Gadella, 2000). Insemination with frozen semen has improved reproductive efficiency as well as economic profit in the goat industry. The objective of the present work was to reinstate the oxidant-antioxidant balance in the system by the addition of catalase in extender for increasing the life and fertility potential of cryopreserved semen.

MATERIALS AND METHODS

All chemicals were obtained from Sigma Chemicals Co. (St. Louis, MO, USA) unless otherwise stated. During the study, all the experimental protocols met the Institutional Animal Care and use committee regulations.

Extender preparation

The basic semen extender was composed of 3.604 g tris, 1.902 g citric acid, 1 g fructose, 200 mg streptomycin, 120 mg penicillin, and 200 mL triple distilled water. The pH was adjusted to 6.8. For preparing the working solution 10% egg yolk (v/v) and 6 % glycerol (v/v) were added freshly to the basic extender and mixed well.

Semen collection and evaluation

Sexually mature bucks (N-12), managed under semi-intensive system were used in the study. Semen samples were collected twice a week using an artificial vagina after stimulating with an oestrous doe. Immediately after collection volume, color, consistency, and mass motility of ejaculates were assessed. Ejaculates with mass motility +4 and above were pooled of the same buck and divided into 6 equal aliquots. The samples were extended with Tris- Citric acid- Fructose diluent having 10% (v/v) egg yolk as an extracellular and 6% (v/v) glycerol as an intracellular cryo protecting agent. In this experiment we used catalase (CAT; 25 mg, 11700 U, SRL) and had 6 groups: control (non-supplement), 200 IU/mL, 400 IU/mL, 600 IU/mL, 800 IU/mL and 1000 IU/mL. The progressive motility, live cell count, functional plasma membrane integrity, acrosome integrity, and mitochondrial membrane potential (MMP) were evaluated immediately after dilution and after 24 h of cryopreservation in liquid nitrogen. Sperm concentrations were adjusted to 1×10^8 mL^{-1} and diluted semen was equilibrated at 5 ˚C for 4 h before being liquid nitrogen frozen (Ranjan *et al.* 2015).

Semen cryopreservation

After equilibration period of 4 h semen was filled in 0.25 ml straws at 5 ˚C and straws were sealed with polyvinyl alcohol powder. After filling and sealing, straws were vapour frozen in liquid nitrogen vapours by keeping them 2 cm above the liquid nitrogen for 10 min. Finally, straws were plunged into liquid nitrogen and stored in a liquid nitrogen container (Gangwar *et al.* 2015).

Motility assessment

Diluted semen (10 µL) was placed on a clean grease-free warm slide (38 ˚C) with cover slip and observed under 400X magnification of phase contrast microscope. The progressive motility was calculated by considering the average values of two independent experts.

Sperm vitality assessment

The live and dead spermatozoa were calculated by using Eosin – Nigrosine stain (Hancock, 1951; Ranjan *et al.* 2009a).

The dead sperm cell takes eosin colour, while those which are alive before staining do not take any colour.

Acrosomal integrity assessment

The acrosomal integrity of spermatozoa was assessed by using Giemsa stain (Watson, 1975; Ranjan *et al.* 2014). A thin smear of diluted semen was prepared on a clean grease free slide and air-dried. The slide was then immersed in Hancock's fixative for 1h at 38 ˚C. The slides then washed in running water and air dried. The slides were then immersed in Giemsa stain (6 mL Giemsa stain, 4 mL Sorrenson buffer and 90 mL triple distilled water) for 2 h at 38 ˚C. After staining, the slides were washed in water and air-dried. The slides were examined for acrosome morphology under the oil immersion objective of the microscope. A total of at least 200 sperms were counted and the percentage of acrosomal alterations was calculated.

Functional membrane integrity assessment

The membrane integrity of spermatozoa was examined by a hypo-osmotic swelling (HOS) test (Revell and Mrode, 1994; Ranjan *et al.* 2009b). Diluted semen (10 µL) was added to 1 mL of hypo-osmotic solution (75 mOS) in microtubes and incubated at 38 ˚C for 2 h. Immediately after incubation, semen (10 µL) was placed on a glass slide, covered with a coverslip, and examined under 400X. A total of at least 200 spermatozoa were counted besides total coiling, a strong coiling was the description given when the tail became much coiled (Ranjan *et al.* 2017).

Fluorogenic assessment of mitochondrial membrane potential

The assessment of MMP was done as described earlier (Selvaraju *et al.* 2008). MMP was assessed by using JC-1 (5,5,6,6-tetrachloro,1,3,3 tetra ethyl benzimidazolocarbocyanineiodide) (Figure 1). Prepared solution (3 µL of 1.53 mM JC-1 indimethyl sulfoxide) was added to a 100 µL frozen-thawed sperm sample (100×10^6/mL), and incubated for 30 min at 38 ˚C. Then, cells were washed once in phosphate-buffered saline (PBS) and resuspended in PBS, smeared and analyzed with epifluorescence microscope (Axiovert, Ziess) using an excitation wavelength of 485 nm.

A minimum of 200 sperms were observed at 400X magnification. The cells with yellowish to orange fluorescence (aggregates) in the mid piece were considered as cells having MMP.

Artificial insemination in estrous goat

Intra-cervical AI was used to get maximum benefits. For Intra cervical AI, the oestrous goat lifted from the back for clear visualization of genitalia. A lubricated glass vaginal speculum was inserted through vagina for visualization of cervical opening under sunlight. Then frozen-thawed semen straw inserted through vaginal speculum and go through cervical opening and semen was deposited there and waits for two to three minutes. 40 goats (20 control and 20 experimental) were inseminated with frozen semen in natural estrous conditions. The insemination was carried out 12 h after detection of estrous and repeated after 12 h of the first insemination.

Transrectal ultrasonography of goats

Trans rectal ultrasonography was carried out using 5/7 MHz transducer in inseminated does with frozen semen at or after 28 days post-mating. Inseminated goats were not given feed for 12 h (overnight) and ultrasonography was done in morning.

Statistical analysis

Statistical analysis was performed by General Linear model of SPSS (SPSS, 2011). The factorial model included the effect of catalase concentration as an independent variable and percent post thawed motility, live sperm count, acrosome intact sperm, hypo osmotic swelling positive sperm and MMP as dependent variables. Post Hoc Test Duncan LSD T3 was conducted to know the significant difference between different variables at $P < 0.05$. A homogeneity test was also conducted to assign different superscripts to variables based on significant difference at $P < 0.05$ between different variables.

RESULTS AND DISCUSSION

Effect of catalase concentration on post-thaw semen quality

The effects of catalase in semen diluent on post-thaw seminal qualities were evaluated and the percentages of motile spermatozoa, live and dead spermatozoa, hypo osmotic swelled spermatozoa, acrosome integrity, and MMP for each concentration was averaged. The progressive motile spermatozoa, live spermatozoa, hypo-osmotic swelled spermatozoa, acrosome integrity percent and MMP were 80.94 ± 1.04, 83.24 ± 1.03, 80.73 ± 1.11, 81.92 ± 1.10 and 70.28 ± 1.13 respectively in fresh semen. The post-thaw semen quality was decreased significantly ($P<0.05$) after cryopreservation. The effect of catalase on post-thaw semen quality was summarized in Table 1. The results showed that the progressive motility, live sperm count, hypo-osmotic swelling positive spermatozoa, acrosomal integrity, and MMP (Mean±Standard error) were significantly higher ($P<0.05$) in 800 IU/mL catalase concentrations (Table 1).

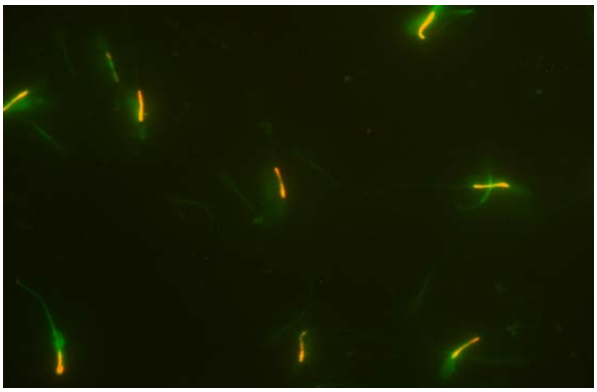

Figure 1 Mitochondrial membrane potential (MMP) of goat spermatozoa evaluated by JC-1
Sperm with low MMP (high green and low orange fluorescence), sperm with high MMP (low green and high orange fluorescence)

Table 1 Effect of catalase on post-thaw semen qualities (Mean±Standard error)

Concentration (catalase IU/mL)	Post thaw motility %	Live %	HOST %	Acrosome %	MMP %
0 (control)	36.25±2.05^{b}	39.95±2.30^{b}	36.53±1.64^{b}	34.69±0.59^{b}	32.00±1.05^{cd}
200	35.63±1.76^{b}	39.93±1.93^{b}	37.35±2.24^{b}	38.88±2.07^{b}	34.14±1.18^{b}
400	34.28±1.30^{b}	38.19±1.64^{b}	37.04±1.39^{b}	37.42±1.94^{b}	30.14±1.10^{d}
600	34.28±1.30^{b}	36.75±1.38^{b}	38.59±2.04^{ab}	39.87±1.38^{b}	31.28±0.81^{cd}
800	42.86±2.86^{a}	47.81±2.29^{a}	44.73±3.48^{a}	46.81±2.50^{a}	39.71±0.56^{a}
1000	32.86±1.01^{b}	39.11±0.71^{b}	37.61±1.50^{b}	40.28±1.83^{b}	30.86±0.86^{d}

MMP: mitochondrial membrane potential.
The means within the same row with at least one common letter, do not have significant difference (P>0.05).

The MMP was significantly higher (P<0.05) in 200 IU/mL catalase concentrations than control and 600 IU/mL catalase concentrations.

Effect of catalase on conception rate

A total 20 goats were chosen for frozen semen AI technique and 8 goats (40%) were conceived by this technique using frozen semen straw of 800 IU/mL catalase as an antioxidant.

The post thaw quality parameters showed significantly high in 800 IU/mL catalase, so, this particular concentration of catalase was compared with control for *in vivo* fertility test. The conception rate in control group was 7 out of 20 goats (35%) (Figure 2).

In the present study, the results revealed that addition of catalase 800 IU/mL has improved the motility, live cell count, membrane integrity, acrosome integrity and MMP of buck semen and thus it protects the structures and functions of spermatozoa efficiently. Catalase has beneficial effects on the preservation of mammalian sperm and improves the functional parameters of spermatozoa (Gungor *et al.* 2018). The incorporation of ascorbic acid, catalase and ascorbic acid + chlorpromazine in semen extender improved the post-thaw semen quality in crossbred bulls (Paudel *et al.* 2010).

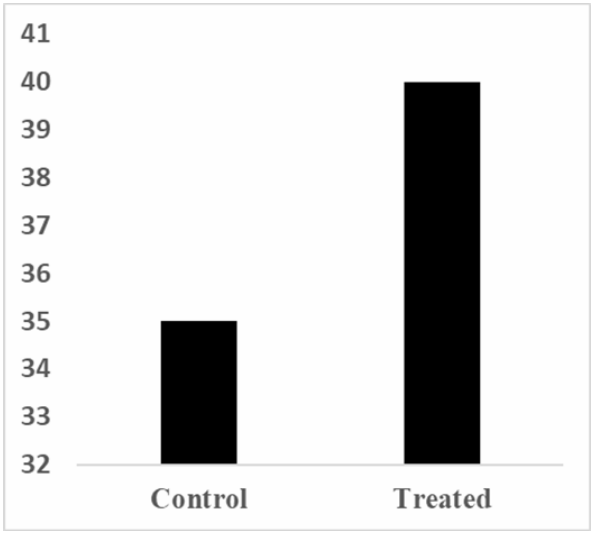

Figure 2 Conception percent by using frozen semen straws having catalse (800 IU/mL) in treated and control animal group

Our results showed that pre-freezing and freezing-thawing of semen significantly decreased the percentages of motility, viability, HOS, acrosomal integrity and MMP. This may be due that ROS are generated more during cryopreservation and freezing and thawing protocols (Chatterjee and Gagnon, 2001).

These ROS increase the susceptibility of spermatozoa to oxidative stress as affected by cold shock (White, 1993), which play important role in decreasing motility, shortening the life span of spermatozoa and affecting the membrane integrity and preservation of semen. This study showed that 800 µM of catalase was more effective in post-thaw quality parameter. This is because catalase activates decomposition of hydrogen peroxide (H_2O_2) into water and oxygen (Fernandez *et al.* 2007). Breakdown of hydrogen peroxide into water and oxygen in the presence of catalase may block the pathways, which generate ROS and thus prevent the loss in motility (Roca *et al.* 2005). Due to its potent antioxidative nature, catalase supplementation decreased oxidative stress and showed its beneficial effects in semen preservation (Thiangtum *et al.* 2012). Catalase causes membrane lipids and proteins rearrangements which results in increased membrane fluidity and thus decreased the percentage of acrosome damages (Holt, 2005; Witte and Schafer-Somi, 2007).

Acrosomal damages were observed during cryopreservation in ram to about 45–65% (Abdelhakeam *et al.* 1991), in goat to about 38–43% (Chauhan *et al.* 1994) and in cattle to 26% (Azam *et al.* 1998) and buffalo was more than 20% (Kumar *et al.* 2016). The reduction in the seminal parameters might be due to the overproduction of ROS from continuously increasing number of dead sperm cells that cannot be checked by catalase as its concentration was fixed. Also, overdose of catalase causes high fluidity of plasma membrane above desired point, making sperm more prone to oxidative stress induced damage (Chen *et al.* 2013).

The mitochondria activity is a key indicator of sperm function. Mitochondria, localized in the mid-piece area of sperm flagellum, produce ATP to support sperm motility. This study observed a significant decrease in high MMP in cryopreserved sperm. Similar finding was observed by various workers in different species (Ly *et al.* 2003; Martin *et al.* 2007; Kadirvel *et al.* 2012). A decrease in MMP lead to a lack of energy, which may be responsible for reduced sperm motility. Hence, reduced sperm motility induced by cryopreservation is believed to be mainly associated with mitochondrial damage (Januskauskas and Zillinskas, 2002).

Supplementation of Tris extender with IGF-I improved subjective sperm motility and structural integrity of the plasma membrane without a significant effect on pregnancy rates of ewes with frozen thawed semen (Padilha *et al.* 2012). It becomes very difficult to pass the AI gun throughout the cervix due to the complex cervical anatomy in goats. Therefore, the conception rate is highly correlated with the depth of penetration. Although the laparoscopic AI involving deposition of frozen-thawed semen directly into the uterus generally results in 60-70% fertility (Salamon and Maxwell, 1995), the conception rate of cryo-preserved semen following trans-cervical AI (TCAI) is still very low (16-40%; Kharche *et al.* 2013; Kumar and Naqvi, 2014).

The Embrapa AI technique resulted in satisfactory rates of cervical transposing and intrauterine AI, achieving reasonable pregnancy rates in goats (Jeferson *et al.* 2017). Inhibition of angiotensin-converting enzyme in goats under protocol of fixed-time artificial insemination improves pregnancy rates, parturition, twinning and proved to be a good alternative for increasing the efficiency of such a biotechnique (Fernandes *et al.* 2018). The premature capacitation as a consequence of freezing and thawing curtails the lifespan of spermatozoa having a very shorter time to achieve fertilization compared to the fresh sperm.

CONCLUSION

We inferred that the addition of catalase (800 IU/mL) significantly reduced the detrimental effects of cooling on motility, viability, membrane integrity, and acrosomal integrity of buck sperm. This particular concentration also showed significant ($P<0.05$) improvement in mitochondrial membrane potential and also we have achieved a significantly higher conception rate in goat. So, catalase (800 IU/mL) can be used semen diluent for routine semen cryopreservation in goat.

ACKNOWLEDGEMENT

The authors are thankful to the Department of Bio-Technology, MoS, New Delhi for financial support and the Director, ICAR-CIRG, Makhdoom for providing all necessary facilities to conduct this experiment.

REFERENCES

Abdelhakeam A.A., Graham E.F. and Vazque I.A. (1991). Studies on the absence of glycerol in unfrozen and frozen ram semen: Fertility trials and the effect of dilution methods on freezing ram semen in the absence of glycerol. *Cryobiology*. **28,** 36-42.

Acharya R.M. (1987). Breeds of goats and research programmes for their in India. Pp. 772-805 in Proc. 3rd Int. Conf. Goats. Brasilia, Brazil.

Aitken R.J. (2017). Reactive oxygen species as mediators of sperm capacitation and pathological damage. *Mol. Reprod. Dev.* **84,** 1039-1052.

Azam M., Anzar M. and Arslan M. (1998). Assessment of post-thaw semen quality of buffalo and Sahiwal bulls using new semen assays. *Pakistan Vet. J.* **18,** 74-80.

Baumber J., Vo A., Sabeur K. and Ball B.A. (2002). Generation of reactive oxygen species by equine neutrophils and their effect on motility of equine spermatozoa. *Theriogenology*. **57,** 1025-1033.

Bilodeau J.F., Blanchette S., Cormier N. and Sirad M.A. (2002). Reactive oxygen species-mediated loss of bovine sperm motil-

ity in egg yolk Tris extender: Protection by pyruvate, metal chelators and bovine liver or oviductal fluid catalase. *Theriogenology*. **57,** 1105-1122.

Chatterjee S. and Gagnon C. (2001). Production of reactive oxygen species by spermatozoa undergoing cooling, freezing and thawing. *Mol. Reprod. Dev.* **59,** 451-458.

Chauhan M.S., Kapila R., Gandhi K.K. and Anand S.R. (1994). Acrosome damage and enzyme leakage of goat spermatozoa during dilution, cooling and freezing. *Andrologia*. **26,** 21-26.

Chen S.J., Allam J.P., Duan Y.G. and Haidl G. (2013). Influence of reactive oxygen species on human sperm functions and fertilizing capacity including therapeutical approaches. *Arch. Gynecol. Obstet*. **288,** 191-199.

Fernandes V.P., Silva M.N.N., Costa A.S., Kunkel D., Junior A.S., Feitosa L.C.S., Muratori M.C.S. and Costa A.P.R. (2018). CE inhibition in goats under fixed-time artificial insemination protocol increases the pregnancy rate and twin births. *Reprod. Domest. Anim.* **53,** 1006-1008.

Fernandez S.M.R., Martinez P.F., Garcia M.V., Esteo M.C., Soler A.J., Paz P., Anel L. and Garde J.J. (2007). Sperm characterstics and DNA integrity of Iberian red deer (*Cervus elephus*) epididymal spermatozoa frozen in the presence of enzymatic and nonenzymatic antioxidants. *J. Androl.* **28,** 294-305.

Flesch F.M. and Gadella B.M. (2000). Dynamics of the mammalian sperm membrane in the process of fertilization. *Biochim. Biophys. Acta*. **1469,** 197-235.

Gama L.T. and Bressan M.C. (2011). Biotechnology applications for the sustainable management of goat genetic resources. *Small Rumin. Res.* **98(1),** 133-146.

Gangwar C., Kharche S.D., Ranjan R., Kumar S., Goel A.K. and Jindal S.K. (2015). Effect of vitamin C supplementation on freezability of Barbari buck semen. *Small Rumin. Res.* **129,** 104-107.

Gungor S., Ata A. and Inac M.E. (2018). Effects of trehalose and catalase on the viability and kinetic parameters of cryopreserved ram semen. *Acta Sci. Vet.* **46,** 1-7.

Hancock J.L. (1951). A staining technique for the study of temperature shock in semen. *Nature*. **167,** 323-331.

Holt W.V. (2005). Fundamental aspects of sperm cryobiology: The importance of species and individual differences. *Theriogenology*. **3,** 47-58.

Januskauskas A. and Zillinskas H. (2002). Bull semen evaluation post-thaw and relation of semen characteristics to bull's fertility. *Vet. Zootech.* **17,** 39-47.

Jeferson F.F., Gilmar P.A., Joanna M.G.S., Maria E.F.O., Viviane LB., Felipe Z.B. and Olivardo F. (2017). Reproductive features and use of an anti-inflammatory drug in estrus-induced dairy goats artificially inseminated in a standing position with cervix immobilization. *Reprod. Biol.* **17,** 268-273.

Kadirvel G., Periasamy S. and Kumar S. (2012). Effect of cryopreservation on apoptotic-like events and its relationship with cryocapacitation of buffalo (*Bubalus bubalis*) sperm. *Reprod. Domest. Anim*. **47,** 143-150.

Kharche S.D., Jindal S.K., Priyadhrashini R., Kumar S., Goel A.K., Ramachandran N. and Rout P.K. (2013). Fertility following frozen semen artificial insemination in Jamunapari goats. *Indian J. Anim. Sci.* **83(10),** 1071-1073.

Kumar D. and Naqvi S.M.K. (2014). Effect of time and depth of insemination on fertility of Bharat Merino sheep inseminated trans-cervical with frozen-thawed semen. *J. Anim. Sci. Technol*. **56,** 8-17.

Kumar D., Kumar P., Singh P., Yadav S.P. and Yadav P.S. (2016). Assessment of sperm damages during different stages of cryopreservation in water buffalo by fluorescent probes. *Cytotechnology*. **68,** 451-458.

Leboeuf B., Restall B. and Salomon S. (2000). Production and storage of goat semen for artificial insemination. *Anim. Reprod. Sci.* **62,** 113-141.

Ly J., Grubb D.R. amnd Lawen A. (2003). The mitochondrial membrane potential (Dwm) in apoptosis; an update. *Apoptosis*. **8,** 115-128.

Maneesh M. and Jayalekshmi H. (2006). Role of reactive oxygen species and antioxidants on pathophysiology of male reproduction. *Indian J. Clin. Biochem.* **21,** 80-89.

Martin G., Cagnon N., Sabido O., Sion B., Grizard G., Durand P. and Levy R. (2007). Kinetics of occurrence of some features of apoptosis during the cryopreservation process of bovine spermatozoa. *Hum. Reprod*. **22,** 380-388.

Padilha R.T., Magalhaes-Padilha D.M. Cavalecante M.M., Almeida A.P., Haan K.T., Gastal M.O., Nunes J.F., Rodrigues A.P.R., Figueiredo J.R. and Oliveria M.A.L. (2012). Effect of insulin-like growth factor-I on some quality trials and fertility of cryopreserved ovine semen. *Theriogenology*. **78,** 907-913.

Paudel K.P., Kumar S., Meur S.K. and Kumaresan A. (2010). Ascorbic acid, catalase and chlorpromazine reduce cryopreservation-induced damages to crossbred bull spermatozoa. *Reprod. Domest. Anim.* **45(2),** 256-62.

Pietta P.G. (2000). Flavonoids as antioxidants. *J. Nat. Prod.* **63,** 1035-1042.

Rahman A.N.M.A., Abdullah R.B. and Khadijah W.E.W. (2008). A review of reproductive biotechnologies and their application in goats. *Biotechnology*. **7(2),** 371-384.

Ranjan R., Goel A.K., Ramachandran N., Kharche S.D., Gangwar C. and Jindal S.K. (2014). Comparison between normal and dual staining technique for evaluating acrosome status and viability in frozen thawed buck spermatozoa. *Indian J. Small Rumin.* **20(2),** 50-53.

Ranjan R., Goel A.K., Ramachandran N., Kharche S.D. and Jindal S.K. (2015). Effect of egg yolk levels and equilibration periods on freezability of Jamunapari buck semen. *Indian J. Small Rumin*. **21(1),** 32-36.

Ranjan R., Priyadharsini R., Goel A.K., Singh B., Kumar S., Kharche S.D. and Jindal S.K. (2017). Effect of membrane stabilizer on the freezability of buck semen. *Indian J. Anim. Sci.* **87(4),** 435-436.

Ranjan R., Ramachandran N., Jindal S.K. and Sinha N.K. (2009a). Effect of egg yolk levels on keeping quality of Marwari buck semen at refrigeration temperature. *Indian J. Anim. Sci.* **79(7),** 662-664.

Ranjan R., Ramachandran N., Jindal S.K. and Sinha N.K. (2009b). Hypo osmotic swelling test in frozen thawed goat spermatozoa. *Indian J. Anim. Sci.* **79(10),** 1022-1023.

Revell S.G. and Mrode R.A. (1994). An osmotic resistance test for bovine semen. *Anim. Reprod. Sci.* **36,** 77-86.

Roca J., Rodriguez M.J., Gil M.A., Carvajal G., Garcia E.M. and Cuello C. (2005). Survival and *in vitro* fertility of boar spermatozoa frozen in the presence of superoxide dismutase and / or catalase. *J. Androl*. **26,** 15-24.

Salamon S. and Maxwell W.M.C. (1995). Frozen storage of ram semen II. Causes of low fertility after cervical insemination and methods of improvement. *Anim. Reprod. Sci*. **38,** 1-36.

Saraswat S., Jindal S.K., Ramachandran N., Yadav S. and Priyadarshini R. (2012). Standardization of antioxidants fortification in frozen buck semen. *Indian J. Small Rumin. Res.* **18(1),** 1-11.

Selvaraju S., Ravindra J.P., Ghosh J., Gupta P.S.P. and Suresh K.P. (2008). Evaluation of sperm functional attributes in relation to *in vitro* sperm-zona pellucida binding ability and cleavage rate in assessing frozen thawed buffalo (*Bubalus bubalis*) semen quality. *Anim. Reprod.. Sci*. **106,** 311-321.

SPSS Inc. (2011). Statistical Package for Social Sciences Study. SPSS for Windows, Version 20. Chicago SPSS Inc., USA.

Thiangtum K., Hori T. and Kawakami E. (2012). Effect of catalase and superoxide dismutase on motility, viability and acrosomal Integrity of canine spermatozoa during storage at 5. *Thai J. Vet. Med.* **42(4),** 447-453.

Watson P.F. (1975). Use of Giemsa stain to detect changes in acrosome of frozen ram spermatozoa. *Vet. Rec*. **97,** 12-15.

White I.G. (1993). Lipids and calcium uptake of sperm in relation to cold shock and preservation. *Reprod. Fertil. Dev*. **5,** 639-658.

Witte T.S. and Schafer-Somi S. (2007). Involvement of cholesterol, calcium and progesterone in the induction of capacitation and acrosome reaction of mammalian spermatozoa. *Anim. Reprod. Sci*. **102,** 181-193.