International Journal of Pharmacy and Biological Sciences
ISSN: 2321-3272 (Print), ISSN: 2230-7605 (Online)
IJPBS | Volume 8 | Special Issue 1- ICMTBT | 2018 | 37-43
International Conference on Microbial Technology for Better Tomorrow ICMTBT
Held at D.Y. Patil College of Arts, Commerce and Science, Pimpri, Pune, 17-19 February
| Conference Proceedings | Research Article | Biological Sciences |Open Access |MCI Approved|
|UGC Approved Journal|

STUDIES ON SEMEN PRESERVATION AND EXPRESSION OF HSPA8 IN ENDANGERED INDIAN LEOPARD (*PANTHERA PARDUS FUSCA*) SPERMATOZOA BY USING QUANTITATIVE PCR (Q-PCR)

PGS Manjusha[1]* and Sadanand D. Sontakke[2]
[1]*Department of Microbiology, Researcher, CCMB (LaCONES)
[2]Principal Scientist and Project Leader, CCMB (LaCONES)
Laboratory for Conservation of Endangered Species (LaCONES), Centre for Cellular and Molecular Biology (CCMB) Annexe-I (CSIR), Attapur Ring Road, Hyderabad 500 048, Tel India.

***Corresponding Author Email:** manjushap53@gmail.com

ABSTRACT

Objective: *The aim of the present study was to carry out leopard semen profiling and devise better ways of semen preservation in order to facilitate effective species conservation. To successfully carry out semen profiling of leopard semen in order to study the various attributes, perform short time cold storage of leopard semen, motility parameters of both fresh and cold stored leopard spermatozoa using a simple microscope. To develop leopard semen cryopreservation protocol to achieve optimum post-thaw viability and to investigate the stability of HSPA8 (Heat Shock Protein A8) in sperms after post-thaw motility by q-PCR, and a gene associated with membrane fluidity in sperm post-thaw by q-PCR. To develop protocols for optimum semen preservation in order to facilitate effective species conservation*

KEY WORDS

Panthera pardus fusca*, Semen Profile, Sperm Motility, Semen Cold Storage, Cryopreservation, miRNA, and qPCR.*

INTRODUCTION:

Biodiversity is important for sustaining a healthy Earth, but it is also immensely valuable to the health and lifestyle of human society. In nature, different species are linked directly or indirectly to various food webs. The disappearance of one species could influence several others down the line. Species which are physically large and those living in forests or oceans are more affected by habitat reduction. Some expert's estimate that around 30% of all species on the Earth will be extinct by 2050. According to the International Union for Conservation of Nature (IUCN), globally about one third of all known species are threatened with extinction. Even it is estimated that 25% of all mammals will be extinct within 20 years.

The Indian Leopard*:** The Indian Leopard ***(Panthera pardus fusca) is leopard subspecies widely distributed on Indian Subcontinent. The species ***Panthera pardus*** is classified as Near Threatened by IUCN since 2008 because populations have declined following habitat loss and fragmentation, poaching for illegal trade of skins and body parts, and persecution due to conflict situations, although its relatively large population size means that it remains one of the most abundant big cat species on the subcontinent. As on September 2015, the leopard population of India itself was estimated between 12,000 and 14,000 individuals.

MATERIALS AND METHODS:

Study area and animals

The present study was conducted between June to December 2016 at the Laboratory for the Conservation of Endangered Species, Hyderabad (78.3 E and 17.2 N), and India. Three adult males (average body weight 80-100 Kg) housed at the Animal Holding Facility at LaCONES were used in the study.

Anaesthesia and Semen Collection

Animals were withheld from food and water 24h prior to the administration of anaesthesia. Anaesthethic procedures were generally conducted between 9 am and 11 am. Animals were administered with a combination of 2.2 mg/kg ketamine hydrochloride (Ketamil 100 mg/ml, Troy Laboratories, Smithfield, New South Wales, Australia) and 1.1 mg/kg xylazine hydrochloride (Ilium Xylazil - 100 mg/ml, Troy Laboratories, Smithfield, New South Wales, Australia). (Sontakke et al. 2009). Both the drugs were mixed together and loaded into a 5 ml projectile plastic syringe dart equipped with a 35 mm needle and darted intramuscularly, in the hindquarter, using a blowpipe as described earlier by Sontakke et al (2009). The animals were left undisturbed to achieve complete anaesthesia. Semen was collected by electroejaculation procedure as described previously (Jayaprakash et al.2001). Immediately after completion of experimental procedures, anaesthetic effects were antagonized with an injection of 0.15 mg/kg yohimbine hydrochloride administered intravenously.

Semen Evaluation

After immediate, collection of semen sample of Leopard was maintained at 37°C in a portable incubator. Volume was determined with a micropipette, whereas pH was determined using colour indicator strips to determine at what concentration of semen was present after ejaculation. Ejaculates were collected separately at the end of each series of stimuli, and semen characteristics such as volume, pH, and percentage of motile spermatozoa were determined. Sperm concentration was evaluated using a computer aided sperm analyser (CASA; HTM-IVOS, Version 10; Hamilton Thorne Research, Danvers, MA, USA; Sontakke et al. 2004) what is the percent of normal live sperms and dead sperms.

Short-Term Cold Storage of Semen

Spermatozoa of wild felids are very sensitive to cold shock because of the high content of polyunsaturated fatty acids in sperm membrane and are therefore susceptible to peroxidative damage with a subsequent motility loss and eventually the sperm function. In preliminary experiments, we used Tes-*n*-Tris extender (TEST) supplemented with egg yolk (TYB). In the second extender, the TYB was supplemented with 8% glycerol. And this media is used for storage of leopard semen. Semen samples were diluted with TYB medium containing different concentrations of egg yolk like (5% and 20%). According to the concentration of spermatozoa in neat semen, then the required dilutions were made and the sample with the extender was then placed in the refrigerator (at ~ 4˚C) in a cryo container for 2 – 3 days. And observe sperm variables such as motility, percentage of sperm abnormality and viability were evaluated over a period of storage.

Semen Cryopreservation and Quantitative PCR (q-PCR)

Fresh semen was analyzed for basic sperm parameters such as motility, viability etc. Seminal plasma was removed after centrifugation of semen at 800 rpm for 7 minutes. The spermatozoa settle at the bottom of the tube as pellet. The supernatant was discarded, and the pellet was resuspended in equal amount of TALP or Ham's F-10 medium supplemented with BSA and pyruvate (Sontakke et al. 2004). This step was repeated twice to obtain spermatozoa without cell debris and free of seminal plasma.

Test-Yolk buffer was used as the buffer system and was prepared by supplementing TEST medium with 20% egg yolk and centrifuged to remove debris. This was divided into two parts and the first part served as the control. To the second part, Trehalose was added in 1:1 ratio. This served as the test treatment. To both test and control, 4% of glycerol was added. Glycerol serves as the cryoprotectant that helps protect cells from damage due to ice crystal formation. This extended semen was then loaded onto 0.5 mL plastic cryo-straws and sealed with polyvinyl powder. The straws were then loaded in a programmable freezer that cooled the semen from 24°C to -80°C in about 100 minutes. After checking the post thaw motility of the semen after cryopreservation the RNA was isolated by using Trizol method, cDNA synthesis was prepared from RNA which was isolated where as a short DNA molecule termed as a oligodeoxynucleotide primer is hybridised to complementary mRNA that allows the reverse transcriptase enzyme (RT) a RNA dependent DNA dependent polymerase to extend the primer and produce a complementary DNA strand. Then samples

are subjected to qPCR whereas Real-time PCR allows precise quantification of specific nucleic acids in a complex mixture even if the starting amount of material is very at low concentration. This is accomplished by monitoring the amplification of a target sequence in real-time PCR using fluorescent technology. How quickly the amplified target reaches a threshold detection level correlates with the amount of starting material present. Heat shock protein A8 (HSPA8) is a highly conserved member of the Hsp70 family, which is expressed in oviductal cells, translocated into oviductal fluid, and becomes attached to the sperm surface during sperm transport.

The present series of experiments was designed to explore the possibility that bovine recombinant HSPA8 might therefore protect leopard spermatozoa during cryopreservation through its beneficial effects on the sperm plasma membrane. If HSPA8 was added to spermatozoa after thawing, outcomes were also biphasic and beneficial effects on viability also improved plasma membrane integrity. Thus, the HSPA8 addition to extender improves the quality of leopard sperm during chilling (viability) and after cryopreservation (number of sperm with damaged acrosomes and "apoptotic-like" changes). The real time PCR was set for the candidate gene: HSPA8.

RESULTS AND DISCUSSION:

Semen Evaluation

The leopards used in the present study had a mean age of 14.35 ± 3.93 years and the mean body weight was 60.45± 4.9 kg. The animals responded to electro-stimulation and the mean volume of the ejaculate was 2.03 ± 0.16 mL with a mean pH of 7.17 ± 0.06 and the sperm concentration of 53.67 ± 4.45. In the ejaculates, 70.83% ± 5.54% of the spermatozoa were found to be motile.

Short-Term Cold Storage of Semen

Cold storage of semen was successfully performed by first determining the sustenance of viable spermatozoa at 4˚C for a period of up to 72 h. In the preliminary experiments, the effect of Test Yolk Buffer (TYB) on sperm viability was evaluated. It was observed that TYB serves as an excellent medium for the preservation of semen for the purpose of short-term storage. Motility was sustained in the sperm samples even after the lapse of 72 h at 4˚C. However, there was a steady decline in the motility over the course of the experiment. According to the results obtained, it was concluded that TYB medium supplemented with 5% egg yolk yielded the best results in terms of maintaining motility, viability and plasma membrane intactness.

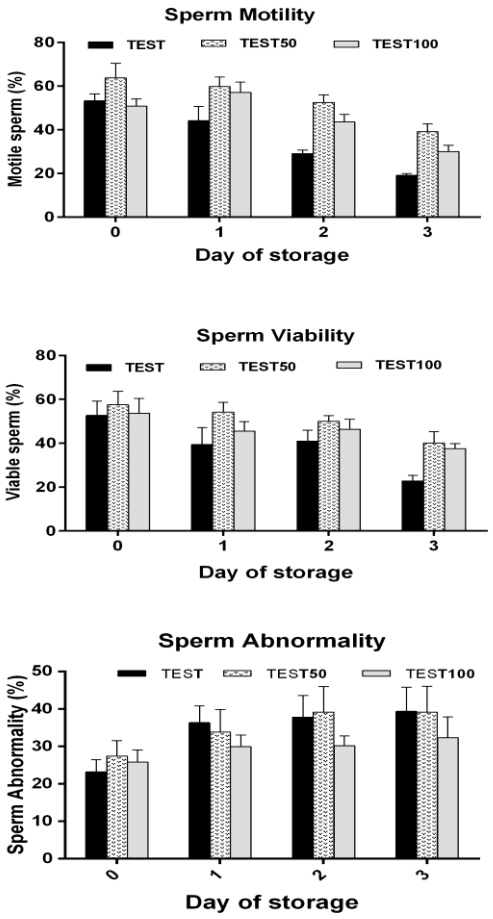


Fig 1: Supplementation of trehalose improves short-term storage of leopard semen. Chart showing the changes in sperm Motility, viability and Sperm Abnormality over a period of storage at refrigerator.

Semen Cryopreservation and Quantitative PCR (q-PCR)

Extensive rounds of preliminary experiments were carried out in order to standardize the optimum concentration of glycerol in the cryo-extender. Although the results were not encouraging, it was observed that 8% glycerol provided maximum cryo-protection as compared to 4 and 6 %. Therefore, for all experiments, the concentration of glycerol used was 8%. Then to improve upon the post thaw sperm recovery in leopard, we supplemented trehalose in two different concentrations (50 and 100 mM). Trehalose at a concentration of 100 mM in the extender also gave slightly similar results with a post-thaw motility of about 15%. All the other concentrations of Trehalose proved

to be unsatisfactory in providing optimum cryo-protection of frozen spermatozoa.

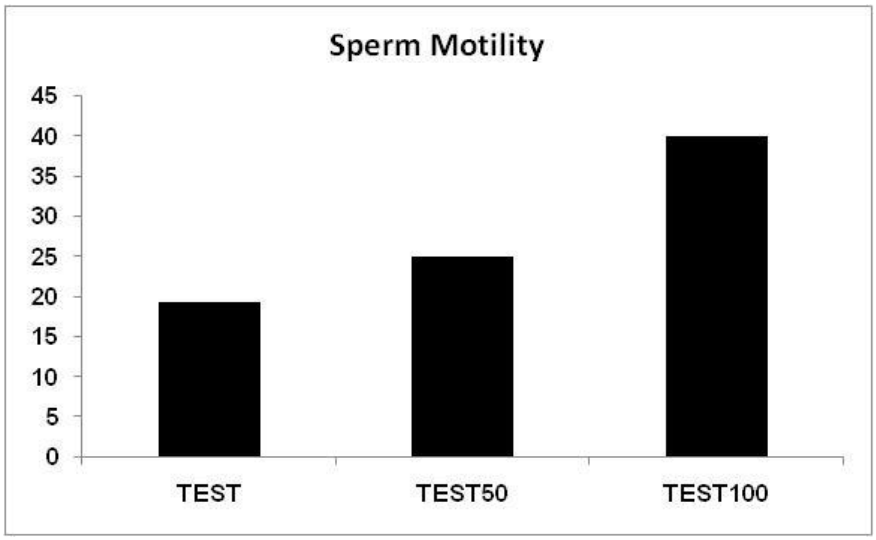
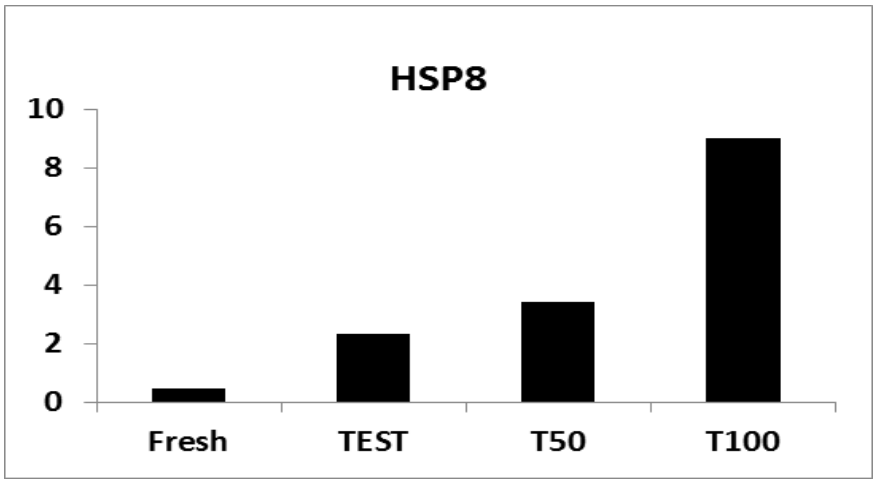


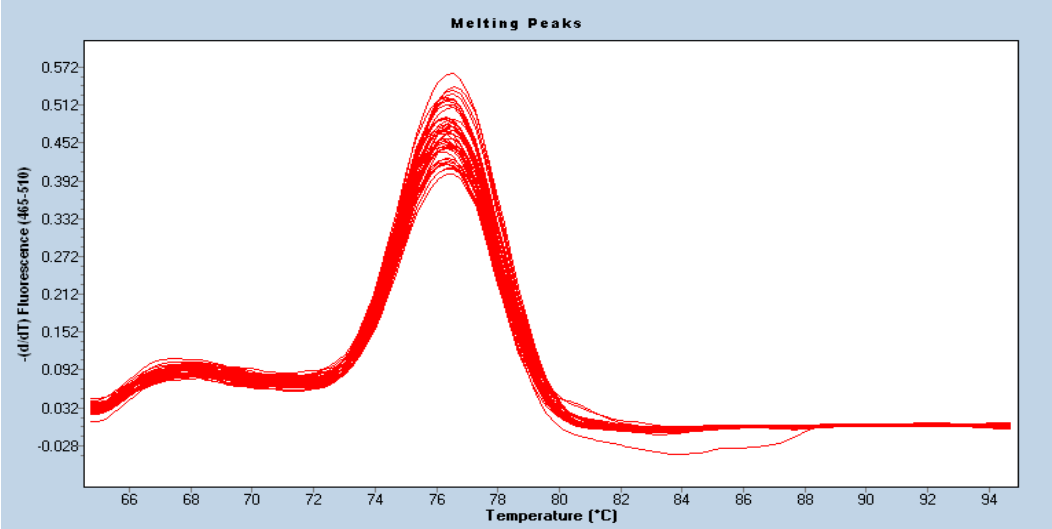
Fig 2: Charts showing effect of glycerol concentration and effect of trehalose concentration on post thaw motility of cryopreserved leopard spermatozoa.

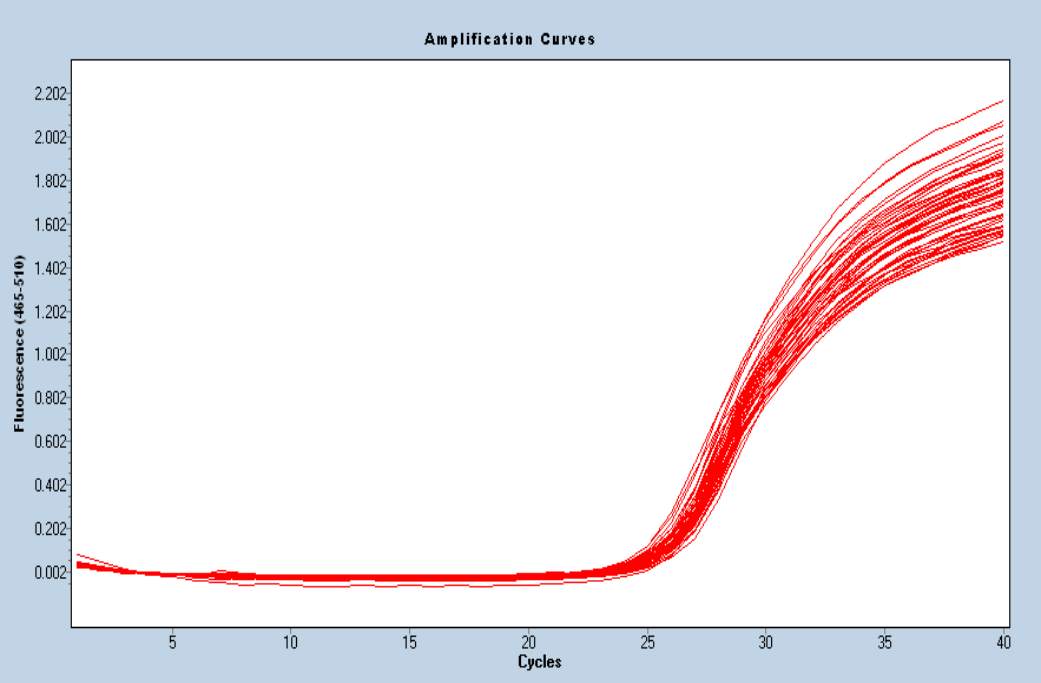


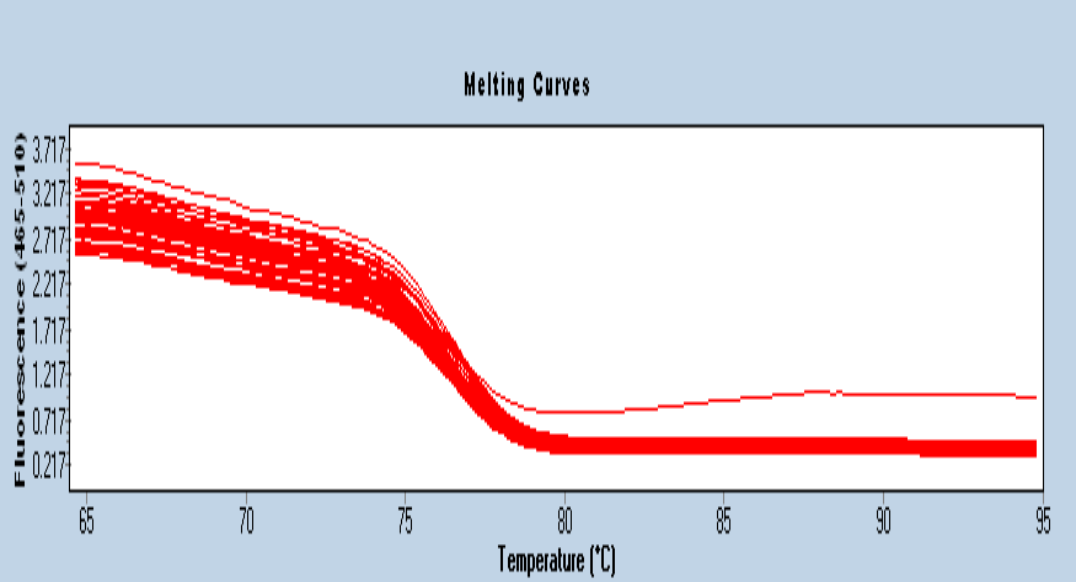
Effect of trehalose supplementation on the expression of HSP8 in leopard sperm during sperm cryopreservation

To determine the reaction efficiency, a standard curve of a chemically synthesized RNA oligonucleotide was generated. RNA was extracted and reverse-transcribed from each aliquot to make cDNA. The levels of HSPA8 in each aliquot were measured by qPCR. The Ct values obtained by qPCR were plotted on a graph for standard curve. The amplification of the PCR product was optimal. A single peak and a melting curve of HSPA8 product was obtained indicating the amplification of a single product without any contamination.

Fig 3: Melting Peaks, Amplification Curves and Melting Curves of HSPA8 by qPCR


Melting Peaks
-(d(dT) Fluorescence (465-510)
0.572
0.512
0.452
0.392
0.332
0.272
0.212
0.152
0.092
0.032
-0.028
66
68
70
72
74
76
78
80
82
84
86
88
90
92
94
Temperature (°C)


Amplification Curves
Fluorescence (465-510)
2.202
2.002
1.802
1.602
1.402
1.202
1.002
0.802
0.602
0.402
0.202
0.002
5
10
15
20
25
30
35
40
Cycles



CONCLUSION:

To the best of our knowledge, this is the first report of successful semen cold storage in wild felids and we also improved the semen preservation procedure by supplementing trehalose in the semen cryoextender. Our experiments suggest that exogenous HSPA8 is a 'rapid response' extracellular cytoprotector and modifier of cell function, which rapidly restores cell membrane integrity by influencing membrane micro viscosity. The study provides information for the first time on the effectiveness of cold storage of semen and cryopreservation of the semen of endangered Indian leopard. This baseline data could be used in captive breeding programs of endangered Indian wild cats. The results are compared and discussed with the available information on other mammals. So, HSPA8 was used to determine the potential presence of any synergistic influence on prolonging sperm viability. HSPA8 was added to spermatozoa after thawing, outcomes were also biphasic and beneficial effects on viability also improved plasma membrane integrity. Thus, the HSPA8 addition to extender improves the quality of leopard sperm during chilling (viability) and after cryopreservation. Successfully, we can preserve leopard sperms by cryopreservation.

FUTURE PERSPECTIVES:

In future, we can use leopard sperms for artificial insemination. By this, we can preserve the leopard species very effectively.

ACKNOWLEDGEMENT:

I would like to express my boundless and great respect to Dr. Sadanand D. Sontakke, Principal Scientist & Project Leader for his patience and for granting me with this work and for all the learning opportunities he provided to me.

REFERENCES:

1. Aboagla, E. E., Terada, T. E. (2004). Effects of egg yolk during the freezing step of cryopreservation on the viability of goat spermatozoa. *Theriogenology* 62, 1160-1172
2. Berlinguer, F., Leoni, G. G., Succu, S., Mossa, F., Galioto, M., Madeddu, M., and Naitana, S. (2007). Cryopreservation of European mouflon (*Ovis gmelini musimon*) semen during the non-breeding season is enhanced by the use of trehalose. *Reprod. Domest. Anim*. 42, 202-207. doi: 10.1111/J.1439-053.2006. 00753.X.
3. Cassinello, J., Abaigar, T., Gomendio, M., and Roldan, E. R. S. (1998). Characteristics of the semen of three endangered species of gazelles. *J. Reprod. Fertil*. 113, 35-45. doi: 10.1530/JRF.0.1130035
4. Donaghue, A. M., Garner, D. L., Donoghue, D. J., Johnson, L. A. (1996). Viability assessment of turkey sperm using fluorescent staining and flow cytometry. *Poultry Science*, 74, 1191-1200
5. Evenson, D. P., Lee, J., Darzynkiewicz, Z., Melamed, M.R. (1985). Rhodamine 123 alters the mitochondrial ultrastructure of cultured L1210 cells. *J Histochem Cytochem.*33, 353–359.
6. Fernandez-Santoz, M. R., Esteso, M. C., Montoro, V., Soler, A. J., and Garde, J. J. (2006). Influence of various permeating cryoprotectants on freezability of Iberian

red deer (*Cervus elaphus hispanicus*) epididymal spermatozoa: effects of concentration and temperature of addition. *J. Andol.* 27, 734-745.

7. Giraud, M.N., Motta, C., Boucher, D., and Grizard, G. (2000). Membrane fluidity predicts the outcome of cryopreservation of human spermatozoa. *Hum. Reprod*. 15, 2160-2164.
8. Holt, W. V., Abaigar, T., and Jabbour, H. N. (1996). Oestrus synchronization, semen preservation and artificial insemination in the Mohor Gazelle (*Gazella dama mhorr*) for the establishment of a genome resource bank programme. *Reprod. Fertil. Dev*. 8, 1215-1222
9. Shivaji, S., Kholkute, S. D., Verma, S. K., Gaur, A., Umapathy, G. (2003). Conservation of wild animals by assisted reproduction and molecular marker technology. *Indian J. Exp. Biol*. 41, 710-723.
10. Sontakke, S. D. (2007). Role of reproductive biotechnologies in the conservation and management of wild animals. In 'You Deserve, We Conserve: A Biotechnological Approach to Wildlife Conservation'. (Eds M. W. Pandit, S. Shivaji and L. Singh.) pp. 29-40. (IK International publishers: New Delhi.)
11. Sontakke, S. D., Reddy, A., Umapathy, G., and Shivaji, S. (2007). Anaesthesia induced by administration of xylazine hydrochloride alone or in combination with ketamine hydrochloride and reversal by administration of yohimbine hydrochloride in captive axis deer (*Axis axis*). *Am. J.Vet. Res*. 68, 20-24. doi: 10.2460/AJVR.68.1.20
12. Sontakke, S. D., Umapathy, G., Patil, M. S., and Shivaji, S. (2009a). Tolazoline antagonizes ketamine-xylazine anaesthesia in an endangered Blackbuck (*Antilope cervicapra*). *Eur. J. Wildl. Res*. doi: 10.1007/S10344-009-0251-X.
13. Sontakke, S. D., Umapathy, G., Sivaram, V., Kholkute, S. D., and Shivaji, S. (2004). Semen characteristics, cryopreservation and successful artificial insemination in the blue rock pigeon (*Columba livia*). *Theriogenology* 62, 139-153. doi: 10.1016/J.THERIOGENOLOGY.2003.08.018.
14. Sontakke, S. D., Umapathy. G., and Shivaji, S. (2009b). Yohimbine antagonizes the anaesthetic effects of ketamine-xylazine in captive Indian wild felids. *Vet. Anaesth. Analg*. 36, 34-41. doi: 10.1111/J.1467-2995.2008. 00427.X.
15. Umapathy, G., Sontakke, S. D., Reddy, A., and Shivaji, S. (2007). Seasonal variations in semen characteristics, semen cryopreservation. Oestrus synchronization and successful artificial insemination in the spotted deer (*Axis axis*). *Therigenology* 67, 1371-1378, doi: 10.1016/J.THERIOGENOLOGY.2007.01.019.

***Corresponding Author:**
PGS Manjusha*
Email: **manjushap53@gmail.com**