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Effect of Storage Temperature on Ram Seminal Plasma and Its Subsequent Use as Epididymal Tail Sperm Recovery buffer

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ABSTRACT

The study was undertaken to assess the effect of storage temperature of ram seminal plasma on its use as recovery and activation buffer for epididymal tail sperm. Five healthy cross bred rams were selected for semen collection maintained at Mountain Research Centre for Sheep and Goats, FVSc & AH, SKUAST-K. Semen was collected by artificial vagina method. Seminal plasma was harvested by double centrifugation method from the semen samples. Recovered seminal plasma was stored at two different temperatures i.e., 4°C and -20°C. Degree of ram epididymal tail sperm activation using homologous seminal plasma stored at 4°C and -20°C was investigated. The spermatozoa recovered in both types of seminal plasma were cold stored separately at 4°C for 72 hours. All the samples were evaluated after every 24 hours for spermatozoa quality. The percentage of sperm motility, viability and HOST reacted populations were significantly higher ($p<0.05$) in seminal plasma stored at 4°C than the seminal plasma stored at -20°C at all the time points of cold storage. Moreover, MDA level of seminal plasma stored at 4°C was significantly ($p<0.05$) lower than the seminal plasma stored at -20°C. In conclusion, seminal plasma harvested for epididymal tail sperm recovery may be stored at 4°C before its subsequent use as epididymal tail sperm recovery and activation buffer. However further studies are required to determine the shelf life of seminal plasma stored at 4°C.

Key words: Epididymal tail sperm, Homologous Seminal Plasma, Temperature, Recovery buffer
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INTRODUCTION

Seminal plasma (SP) is an intricate physiological blend of fluids originating from the testis, epididymis, and accessory sex glands within the male reproductive tract. It plays various roles, such as influencing sperm activity, enhancing their capacity to generate energy through substrates present in the SP, engaging with the epithelia and secretions of the female genital tract, and acting as a signalling carrier for the female, especially regarding her immune system (Alvarez-Rodríguez and Martinez-Pastor, 2021). Although spermatozoa encounter SP for a brief duration, it was initially perceived as a mere transport medium; however, it is now recognized for its significant impact on various mammalian sperm functions. Proteins present in seminal plasma serve to safeguard sperm in both the epididymis and the female reproductive system after ejaculation (Kraus *et al.,* 2005). Ram seminal plasma contains homologous proteins known as ram seminal vesicle proteins (RSVP), with RSVP14 playing a pivotal role by binding to the sperm's plasma membrane and aiding in capacitation (Bergeron *et al.,* 2005; Souza *et al.,* 2012; Leahy *et al.,* 2019). The generation of excessive reactive oxygen species (ROS) during cold storage and cryopreservation leads to oxidative stress (Banday *et al.,* 2017), resulting in the compromise of structural and functional integrity of membranes, increasing membrane permeability, DNA structural damage, and cell death (Gadea *et al.,* 2004; Hsieh *et al.,* 2006). SP is equipped with a robust antioxidant enzyme system, including reduced glutathione (GSH), glutathione peroxidase (GSH-PX), catalase (CAT), and superoxide dismutase (SOD), which counteracts the adverse effects of excessive ROS (Dacheux *et al.,* 2006). Numerous studies highlight the positive influence of SP supplementation on sperm motility, capacitation status, and the ability to penetrate cervical mucus in frozen-thawed ram spermatozoa (Graham 1994; Maxwell *et al.,* 1999; Leahy *et al.,* 2010). In routine assisted reproductive technology, semen processing diminishes sperm fertilization capacity. The detrimental effects of sperm processing can be mitigated by incorporating SP or its components (Muino-Blanco *et al.,* 2008). The present study was undertaken to assess the effect of storage temperature (refrigeration and -20°C) of ram SP on its use as recovery and activation buffer for slaughter house derived epididymal tail sperm.

MATERIALS AND METHODS

Collection of ram seminal plasma: Healthy crossbred breeding rams (n=5) with proven fertility (2-5 years age) were randomly selected from Mountain Research Centre for Sheep and Goat (MRCS&G), Faculty of Veterinary Science and Animal Husbandry, Shuhama, Alusteng, Jammu and Kashmir during breeding season (September-January). The rams were under uniform nutritional (200g concentrate/head twice a day and grazing), housing and environmental conditions. Semen was collected twice (morning and evening) a day for three days per week followed by sexual rest during the next week by artificial vagina. Every day the collected ejaculates were examined grossly for any abnormality followed by microscopic examination. Ejaculates that showed ≥3 mass motility score and ≥70% initial motility were considered as acceptable for harvesting of SP and thus were pooled (SP collected from all rams were pooled). The pooled ejaculate was centrifuged twice, first at 3000 g for 20 minutes and then at 3600 g for 30 minutes to ensure that the SP was free from any sperm. A third centrifugation cycle was followed at 3600g for 30 minutes if sperms were spotted under microscope, Microbial growth during storage was checked by addition of Streptopenicillin @ 1mg/ml and Penicillin @1000 IU/ml. Around 50 ejaculates were collected during this phase yielding 25 ml of SP. On each day of semen collection, SP was divided into two aliquots of 0.5ml each and was stored at 4°C and -20°C, respectively, till further utilization in the experiment (1 month).

Collection of epididymal sperms: The testicles (n=6) were collected from freshly slaughtered rams from the local slaughter house and were transported to the laboratory in an ice chest (4°C-5°C) (Lone *et al.,* 2012) in about 30 min in zip lock bags. Testicles were processed within one hour of slaughter. A small amount of semen was extracted from each testicle with the syringe and was put on warm slide. A drop of Tris buffer was added and examined at 400 magnification of a phase contrast microscope (Eclipse E200, Nikon, Japan). Appearance of vigorous motile sperm was considered as testicle being fertile. Epididymal tail was dissected with a sharp blade into two halves and placed in two small (35mm) petridishes, one containing 2 ml of 4°C stored SP and another containing -20°C stored SP. These dishes were designated as T_4 and T_{20} and were kept around 25 °C to 30°C for 20 minutes to allow the sperm to swim up into the SP for maximum sperm activation. After 20 minutes, pieces of cauda were removed and discarded. Each harvested semen sample was further divided into two aliquots. One aliquot from each group was extended with Tris extender in the ratio of 1:2 i.e. 1 part of SP and 2 parts of Tris extender and other aliquots were kept as unextended. Aliquots were marked as T_4-U, T_4-E, T_{20}-U and T_{20}-E, respectively. A sample (100µl) from all aliquots were kept in 0.5ml micro-centrifuge tube at 30°C for sperm quality evaluation at 0 hour and rest of the samples were kept in the refrigerator for subsequent evaluation at 24, 48 and 72 hours. Sperm quality was assessed for motility

(Mortimer, 1986), viability (Zemjanis, 1970), acrosomal status (Watson, 1975) and functional integrity of sperm plasma membrane by Hypo Osmotic Swelling Test (HOST) (Vasquez *et al.*, 2013).

Measurement of lipid peroxidation: Lipid peroxidation (LPO) of the stored seminal plasma (both 4 and 20 degrees centigrade) was measured at 0 hour only but before using it for sperm recovery process. LPO was determined on the basis of Malondialdehyde (MDA) level using thiobarbituric acid (TBA) method as per the technique described by Rao *et al.*, (1989).

RESULTS AND DISCUSSION

The values for percent sperm motility of sperm sample activated in seminal plasma stored at 4°C and -20°C are presented in the Fig. 1.

The percent sperm motility in seminal plasma stored at 4°C was significantly ($p<0.05$) higher than the seminal plasma stored at -20°C at all the time points of cold storage in unextended group. In extended group, percent sperm motility in seminal plasma stored at 4°C was significantly ($p<0.05$) higher than the seminal plasma stored at -20°C at 0 and 72 hour only. At 24 hour, the live sperm percent of seminal plasma stored at 4°C tended to be significantly ($p<0.059$) higher at 48 hour. Further within the groups, percent sperm motility declined significantly ($p<0.05$) from 0 to 72 hours of cold storage. The values for percent live sperm sample activated in seminal plasma stored at 4°C and -20°C are summarized in the Fig. 2.

The percent live sperm recovered in seminal plasma stored at 4°C was significantly ($p<0.05$) higher than the spermatozoa recovered in seminal plasma stored at -20°C

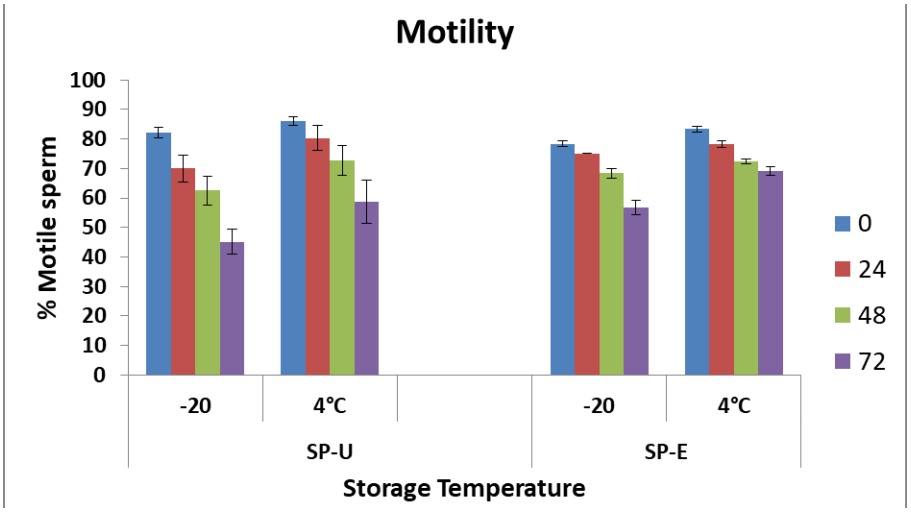

Fig. 1: Sperm motility percentage (Mean±SEM) of sperm sample recovered in seminal plasma stored at 4°C and -20°C.

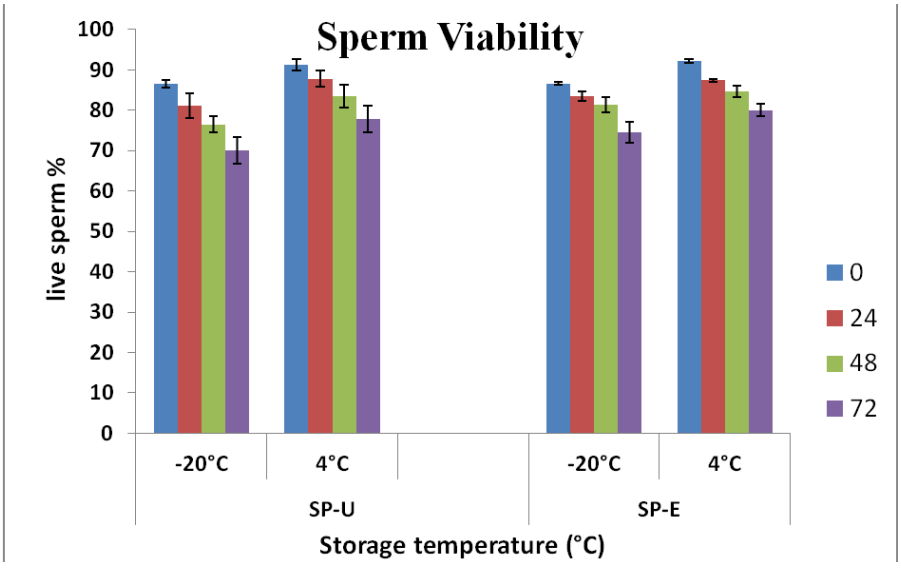

Fig. 2: Viability percentage (Mean±SEM) of sperm sample recovered in seminal plasma stored at 4°C and -20°C.

at all the time points of cold storage in unextended group. In extended group, percent live sperm recovered in seminal plasma stored at 4°C was significantly ($p<0.05$) higher than the spermatozoa recovered in seminal plasma stored at -20°C at 0, 24 and 72 hour of cold storage. Further within the groups, the percent live sperm declined significantly ($p<0.05$) from 0 to 72 hours of cold storage. The percent HOST reacted sperm activated in seminal plasma stored at 4°C and -20°C is set out in the Fig. 3.

The percent HOST reacted sperm for seminal plasma stored at 4°C was significantly ($p<0.05$) higher than the seminal plasma stored at -20°C at all the time points of cold storage in the unextended group. In the extended group the HOST reacted sperm percentage for seminal plasma stored at 4°C was significantly ($p<0.05$) higher than the seminal plasma stored at -20°C only at 0 hour of cold storage only. Further, HOST reacted sperm percentage declined significantly ($p<0.05$) from 0 hour to 72 hour of cold storage. The percent intact acrosome of sperm sample activated in seminal plasma stored at 4°C and -20°C is summarized in the Fig. 4.

The Intact acrosome percentage for seminal plasma stored at 4°C was significantly ($p<0.05$) higher than

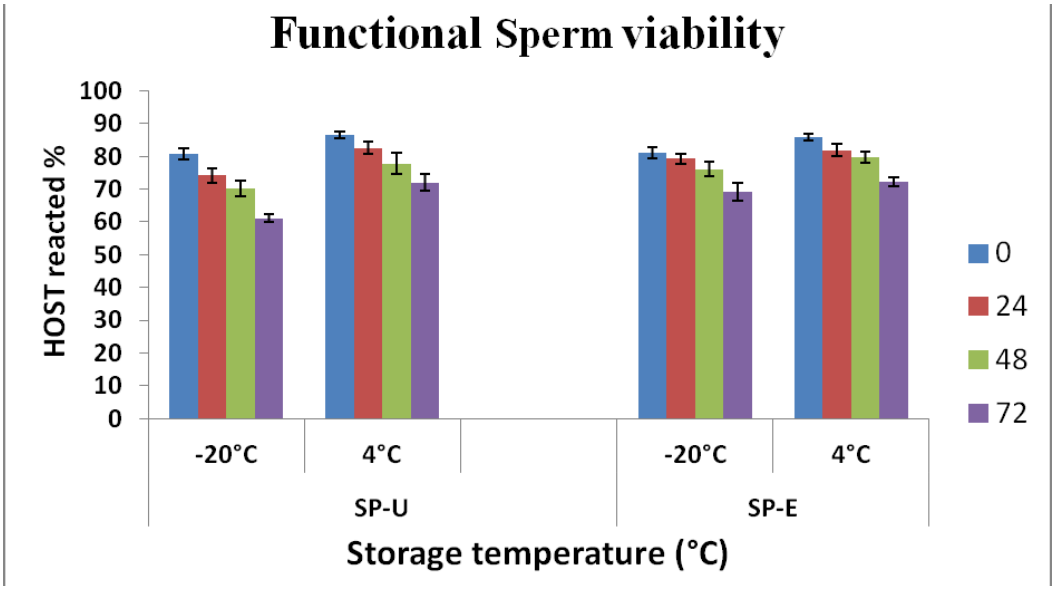

Fig. 3: HOST reacted sperm percentage (Mean±SEM) of sperm sample recovered in seminal plasma stored at 4°C and -20°C.

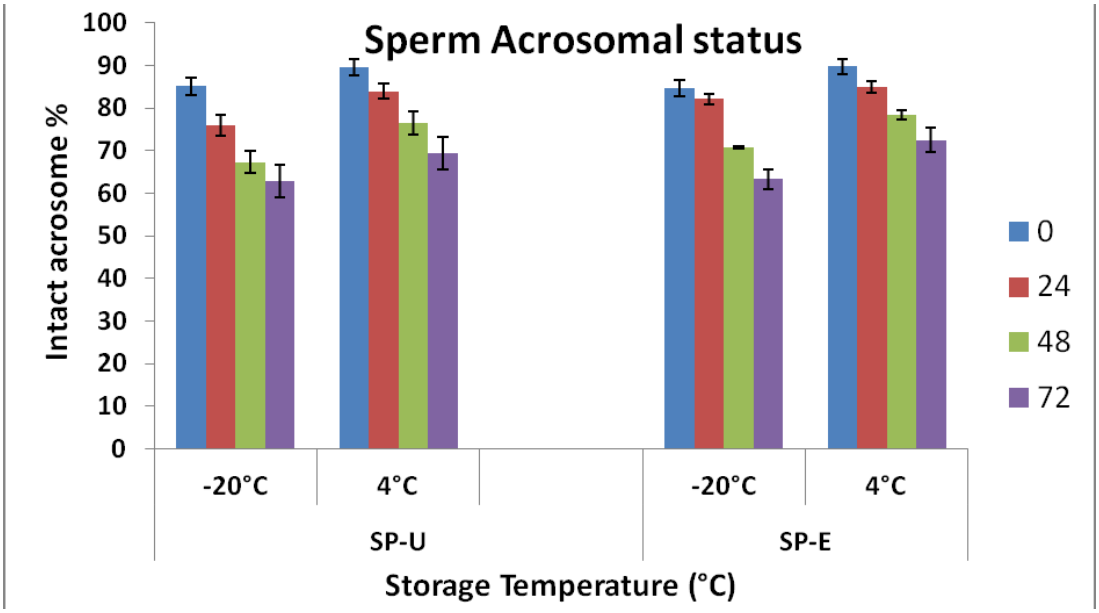

Fig. 4: Acrosomal status (Intact acrosome) percentage (Mean±SEM) of sperm sample recovered in seminal plasma stored at 4°C and -20°C.

the seminal plasma stored at -20°C only at 72 hour in unextended group. Further, intact acrosome percentage declined significantly ($p<0.05$) from 0 hour to 72 hour of cold storage. The MDA level was measured at 0 hour only. The values of MDA level are set out in the Fig. 5.

The MDA level of seminal plasma stored at 4°C was significantly ($p<0.05$) lower than the seminal plasma stored at -20°C.

After collection of seminal plasma, it is necessary to store it till it is used. Generally, all biological fluids are stored either at -20°C or -80°C to prolong their shelf life with less variation in composition. Storing a biological fluid at -20°C or -80°C needs a robust, strong and constant power supply backup. Repeated freeze-thawing has been reported to enhance generation of oxygen free radicals leading to lipid peroxidation (Shaygannia *et al.,* 2020). Risk of lipid peroxidation is more when seminal plasma happens to be stored at -20°C owing to the presence of higher amounts of unsaturated fatty acids (Diaz *et al.,* 2016; Wood *et al.,* 2016). Lipid peroxidation due to freeze-thawing cycles is unlikely at 4°C. We hypothesized that storing seminal plasma at 4°C and using it for sperm recovery might be comparable to seminal plasma stored at -20°C in activating the epididymal tail sperm. With this hypothesis the first objective was to compare abilities of seminal plasma stored at 4°C and -20°C to effectively activate epididymal tail sperm and preserve quality during cold storage upto 72 hours.

All the quality parameters like sperm motility, viability, functional membrane integrity and acrosomal integrity were significantly higher for seminal plasma stored at 4°C compared to seminal plasma stored at -20°C. No published data is available for comparison between the storage temperatures of seminal plasma before its use. The superior sperm quality reported here for seminal plasma at 4°C may be ascribed to significant lower levels of MDA obtained for seminal plasma stored at 4°C compared to seminal plasma at -20°C. An interesting finding in the present study was that MDA levels were higher ($p<0.05$) for seminal plasma stored at -20°C indicating higher lipid peroxidation. This could be the reason for slight degradation of sperm quality in seminal plasma stored at -20°C. Higher MDA levels obtained are attributed to sudden direct power supply breakdown for about three days during the study. Freeze thawing cycle results in high production of ROS leading to lipid peroxidation (Shaygannia *et al.,* 2020). As seminal plasma is very rich in fatty acids, lipid peroxidation resulted in increased levels of MDA reported in this study. On the other hand, storage at 4°C does not involve freeze thawing cycle, hence less level of MDA was obtained. Decreasing trend in the sperm quality from 0 to 72 hours in both extended and unextended group is attributed to the lack of fructose in seminal plasma and the extender (Rather *et al.,* 2016) which degrades with time during the cold storage. No relevant literature to compare the results exists in the literature.

Serum and plasma are also rich in fatty acids but are still stored at -20°C to increase their shelf life. These biological fluids are used for estimation of various constituents rather than used as a medium for cell survival, whereas, seminal plasma is used for sperm survival during storage. There will be bacterial growth in serum and plasma if stored at

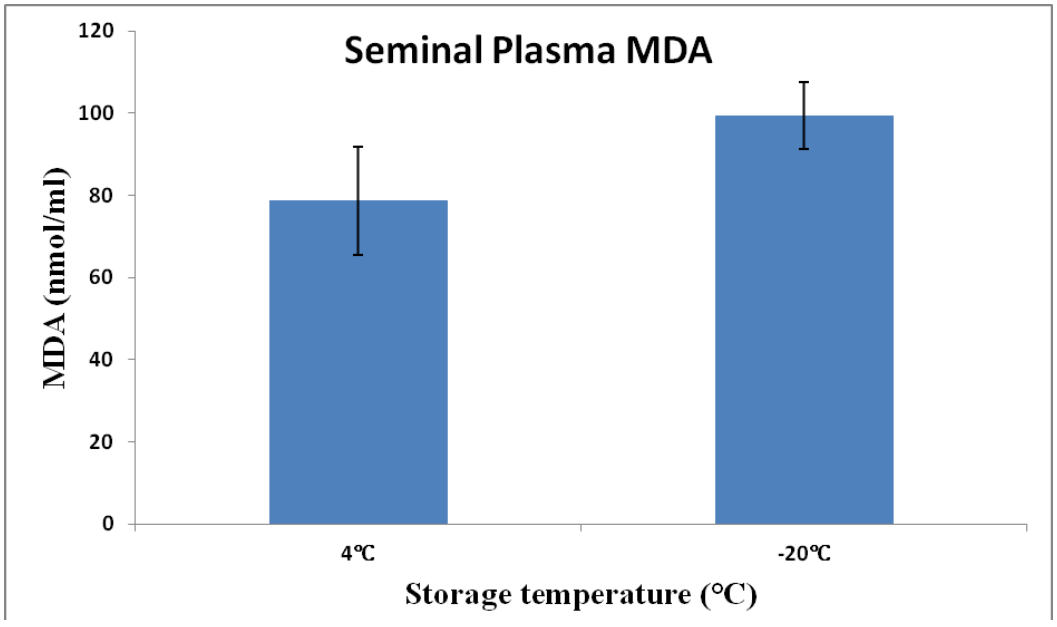

Fig. 5: MDA levels (Mean±SEM) of sperm sample recovered in seminal plasma stored at 4°C and -20°C.

4°C as they are not supplemented with antibiotics at the time of storage while as seminal plasma stored here contained antibiotics to limit bacterial growth. It is concluded that seminal plasma harvested from semen ejaculates may be stored at 4°C rather than -20°C under circumstances where there is no strong and consistent power supply in laboratories. Storing seminal plasma at 4°C is more convenient than at -20°C as refrigerators are easily available in laboratories. Further studies are required to compare the storage temperatures under strong and consistent power supply backup system and also to determine the shelf life of seminal plasma stored at 4°C.

CONCLUSIONS

Seminal plasma harvested for epididymal tail sperm recovery may be stored at 4°C before it is utilized. However further studies are required to determine the shelf life of seminal plasma stored at 4°C.

CONFLICT OF INTEREST

The authors declare that they do not have conflict of interest.

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