EFFECT OF BREEDING AND NON BREEDING SEASONS ON POST THAW QUALITY OF STALLION SEMEN*

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ABSTRACT

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Present study was aimed to compare the different cryoprotective agents for semen of Indian stallions during the breeding season (BS) and non breeding season (NBS). Basic extender used was INRA 82 added with 2 per cent egg yolk. The freezing extenders contained the basic extender added with any of the three different cryoprotectants viz dimethyl formamide (DMF), glycerol (GLY) and ethylene glycol (EG) added singly or in combination. The freezing extender E1 contained 2% DMF, E2 contained 2% GLY, E3 contained 2% EG and extender E4 contained 1% DMF + 1% GLY. Semen was thawed at 60°C for 8 sec and post thaw per cent motility (M), viability (V) and positive sperms to HOST (H) was assessed.

The minimum M was found in E1 (26.51 ± 01.20 %) and E2 (36.38 ± 2.07 %) in BS and NBS respectively, while maximum M was found in E4 (28.74 ± 1.39 %) and E1 (39.12 ± 02.08 %). The minimum V was found in E1 (22.63 ± 01.19 %) and E3 (29.72 ± 01.52 %) in BS and NBS respectively, while maximum was observed in E4 (26.52 ±1.23%), and E2 (32.83 ± 01.67 %). Minimum H was found in E2 (28.60 ± 01.36 %) and (46.00 ± 02.11 %) in BS and NBS respectively, however, maximum H was found in semen cryopreserved in E4 (32.63 ± 01.01 %) and (49.03 ± 01.96 %). Overall extender E4 proved best for cryopreserving stallion semen in Indian conditions followed by E1. While comparing the seasons, the overall values for M, V & H were significantly higher in NBS as compared to BS

Keywords: Stallion semen, Cryopreservation, Cryoprotectants and Seminal attributes, Breeding season.

The use of frozen-thawed semen in horses has increased greatly over the past few years as the advantages of breeding equines with frozen semen are many. Currently accepted standards for frozen equine semen vary around the world, but most commonly set is a minimum level of 25 or 30% post-thaw motility with 200 million motile sperm per insemination is used generally (Katila *et. al.*, 2002).

One of the problems in stallion semen freezing is that not all stallions have sperm that will survive freezing and thawing processes. Roughly 30% of stallions have sperm that will result in good quality thawed semen; further 40% will have "acceptable" post thaw results, and 30% will have sub-fertile frozen spermatozoa. In other words semen of 70% of stallions is capable of achieving pregnancies after freezing.

The main constraint is the stallion to stallion variation in freezability of semen (Davis-Morel, 1999) and non-availability of single technique which can be used for majority of stallions. The reason for this individual stallion dependence is not known. Equine seminal plasma is detrimental to sperm's well being over an extended period of time (Nishikawa, 1975). Some horse's seminal plasma seem to be more of a problem than others, and it is hypothesized that this is main reason for variability of success rates seen from stallion to stallion (Martin *et al.*, 1979). To access individual stallion performance, the spermatozoa

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of such stallion be evaluated using several freezing protocols to identify the best for that stallion (Mc-kinnon and Voss, 1993). Success in the cryopreservation depends on complex series of interactions among the extenders, cryoprotectants, cooling and warming rates to minimize the damage from cold shock, formation of ice crystals and dehydration.

GLY is being used in almost all conventional freezing extenders for equine spermatozoa, but has been reported to be a limiting factor for fertility in horses (Bessa *et al.*, 2006). Different amides are in use as cryo-protective agents, but recently dimethyl-formamide,ethylene glycol and DMSO have been recommended as cryoprotectants to surmount the contraceptive effects of GLY (Vidament *et al.*, 2002). Most of the extenders are egg yolk based which has protective effects on sperm during cooling and freezing (Klug, 1992; De-Vries, 1993).

Frozen semen is a comparatively new technology in equine breeding and as with any new technology, until its practices are standardized, there is need to do further research. The current study was aimed to compare the post-thaw motility, sperm viability and membrane integrity after freezing equine semen using different cryoprotective agents in BS and NBS.

MATERIALS AND METHODS

Five standard bred Indian horse stallions between 5 and 10 years of age at Equine Breeding Stud, Babugarh Distt. Ghaziabad, India at 77.12° E longitude and 28.38° N latitude were used for the study. The stallions were kept under ideal managemental conditions, were weighing more than 500 kgs and housed in quality stables. All the stallions were being given four concentrate feeds (approximately 5.0 kg total) consisting of gram, barley and wheat bran along with *al-lib* green and dry fodder with adequate daily exercises. Semen was collected in two seasons i.e. breeding season (February – October) and non-breeding season (November – January). First five ejaculates of the stallions in each season were discarded to minimize the extra gonadal sperm reserve and were not taken into study. In all seventy (70) ejaculates were collected from five stallions (fourteen ejaculates from each stallion i.e. seven in BS and seven in NBS) using artificial vagina (INRA model) after stimulating stallion on estrous mare. The semen was collected on alternate days between 9.00 and 10.00 a.m. in summer as well as in winter months. The temperature and pressure of artificial vagina (AV) was maintained at 42°C by filling it with warm water. The lubrication was done with non-spermicidal jelly (sterilized Vaseline) applied on inner surface of artificial vagina.

The semen was collected in clean, dry, heat sterilized graduated semen collecting bottles attached with inner latex lining. After collection and removal of gel fraction (in line gel filter) the semen was subsequently maintained at 37°C in a water bath. The ejaculated semen was evaluated for volume, colour, mass motility, concentration, viability and membrane integrity. The volume of semen was directly measured in milliliter(ml) in the graduated semen collecting bottles. Semen samples were also observed for colour and consistency by direct visualization with naked eye and any abnormality in colour and consistency was treated as abnormal and such samples were discarded. The gross motility of neat semen was scored under the low power (10x and 40x) of phase contrast microscope by examining a small drop of semen on a warm slide placed on thermostatically controlled warm stage (37°C) of microscope. The motility was assessed in terms of per cent motile sperms. The sperm concentration was determined on a spectrophotometer (Accucell, IMV technologies, France) duly calibrated for equine semen. Subsequent calibration was done in laboratory through hemocytometer technique (Tomar,1968). For counting live and dead spermatozoa, eosin-nigrosin staining technique as described by Dott and Foster (1972) was adopted. The staining mixture was prepared by taking sodium citrate dehydrate 3.0 gm, nigrosin 5.0 gm and eosin B 1.0 gm in 100 ml of Millipore water. To stain neat semen 4 drops of stain solution were taken in a specimen tube and placed in water bath at 30°C. Then one drop of neat semen was added to the tube containing staining solution, after

two minutes of incubation, thin smears were prepared from this semen stain mixture on a clean and dry slide in air. These slides were seen for live and dead spermatozoa count under oil immersion power of the microscope.

The evaluation of membrane integrity was done with the help of Hypo Osmotic Swelling Test (HOST) as depicted by swelling in tail in 100 mosmol/l of fructose and sodium citrate solution (Pant *et al.*, 2002).In a glass vial 25 µl of fresh semen was mixed with 1.0 ml of hypo osmotic solution (fructose 0.99 gm, tri sodium citrate 0.49 gm in millipore water to make volume 100ml and osmolarity 100 mosm). A small drop from this sample was placed on a clean, dry, grease free glass slide with cover slip and a total of 200 spermatozoa in different fields were observed in a thermostatically controlled stage of phase contrast microscope (200x magnification). The various degrees of hypo osmotic swelling were recorded as recommended by Jeyendran *et. al.* (1984). The spermatozoa showing positive response (coiling of sperm tail) and no response (not showing hypo osmotic swelling) among the 200 spermatozoa counted were used to obtain percentage of HOST positive spermatozoa. The semen was extended up to 200 ml in INRA 82 (basic extender) equine semen extender (Davis-Morel, 1999) and centrifuged at 22°C at 600 g for 10 min to make the pellet of sperms. The supernatant was discarded and pellets were re-suspended in freezing extender (Secondary Extender having INRA 82 + 2% Egg Yolk) making the final concentration of sperms as 100 million/ml. It was divided in four equal parts and added with the cryoprotectants in designated amount. Overall there were four freezing extenders;

E1 = INRA 82 + 2% Egg Yolk + 2% Di Methyl Formamide, E2 = INRA 82 + 2% Egg Yolk + 2% Glycerol, E3 = INRA 82 + 2% Egg Yolk + 2% Ethylene Glycol and E4 = INRA 82 + 2% Egg Yolk + 1% Glycerol + 1% Di Methyl Formamide

The semen diluted in four extenders was kept in cold handling unit for 1 hour and 15 minutes for equilibration and gradual cooling to 4°C. Following equilibration, the semen was filled in French medium straws of 0.5 ml capacity (IMV, France) manually and arranged on a pre cooled stainless steel tray for vapour freezing at -140 °C for 12 min. Subsequently they were plunged into goblets filled with LN_2 and stored in canisters in LN_2 containers. After minimum 24 hours of freezing, semen straws were thawed at 60°C for 8 sec and analysed for post thaw motility, viability and membrane integrity as described above. All the chemicals used were of Sigma Aldrich grade. Statistical analyses of the data were done using students 't' test and ANOVA (Snedecor and Cochran, 1971).

RESULTS AND DISCUSSION

The relevant data for sperm motility (%), viability (%) and per cent positive sperms to HOST (%) for semen frozen during breeding season and non-breeding season and thawed at 60°C for 8 seconds in different extenders are presented in Table 1.

During the BS, the minimum M was found in extender E1 (26.51 ± 01.20 per cent) while maximum M was found in extender E4 (28.74 ± 1.39 per cent) while during non breeding season minimum M was found in extender E2 (36.38 ± 02.07 per cent) while the maximum M was found in extender E1 (39.12 ± 02.08 per cent). Irrespective of the extenders, the overall M during BS was 27.59 ± 00.72 per cent whereas in NBS it was significantly higher 38.23 ± 00.95 per cent.

During the BS, minimum V was found in extender E1 (22.63 ± 01.19 per cent), while maximum was observed in extender E4 (26.52 ± 01.23 per cent) however, during NBS minimum V was found in extender E3 (29.72 ± 01.52 per cent) and maximum was observed in extender E1 (32.83 ± 01.67 per cent). Irrespective of the extenders the overall V during breeding season was found as 24.70 ± 0.69 per cent whereas in non breeding season it was found significantly higher at 30.86 ± 0.78 per cent.

During BS, minimum H was found in extender E2 (28.60 ± 01.36 per cent) and the maximum H was found extender E4 (32.63 ± 01.01 per cent) however, during NBS, the minimum H was found in extender E2 (46.00 ± 02.11 per cent) and maximum H was found in extender E4 (49.03 ± 1.96 per cent). Comparison (paired 't' test) for per cent H revealed that in all the extenders the H was significantly higher (47.36 ± 01.03 per cent) in the NBS as compared to the BS (31.04 ± 00.64 per cent). ANOVA revealed significant difference ($P < 0.05$) in post thaw HOST value between extenders during breeding season, while there is no significant difference in post thaw motility and viability. ANOVA revealed no significant difference ($P < 0.05$) in post thaw motility, viability and HOST value between extenders during non breeding season.

A successful cryopreservation depends upon many factors that involves proper extension of semen, concentrating sperm cells using centrifugation, resuspension of the concentrated cells into extenders containing cryoprotectants, interaction between cryoprotectants, extenders, cooling rate, warming rate, packaging and above all the individual stallion variations (Graham, 1996). Even under ideal conditions some damage to spermatozoa occurs during freezing process. The main damage to sperm cells occurs due to formation of internal ice crystals, which alters the sperm structure due to change in internal solute concentration (Graham,1996).

Going through the results of various extenders, our study revealed that within the extenders, the parameter studied varied significantly between BS and NBS. In all the extenders, the parameters under study were significantly higher In NBS as compared to BS. ANOVA revealed significant ($P < 0.05$) difference between extenders in HOST during breeding season, while there was no significant difference between extenders in post thaw motility and viability. During non breeding season post thaw parameters were non significantly different. The temperature has a significant effect on spermatogenesis. Elevation of testicular temperature due to rise in body temperature or due to an increase in environmental temperature will result in an elevation of the temperature of germinal epithelium and hence have direct adverse effect on spermatogenesis. It has been reported that summer temperature, if increased above 41.5°C for only two hours may be responsible for a depression in spermatogenesis in horses (Johnson, 1991). In northern India, ambient temperature in BS especially in May and June months is more than 41.5°C and hence the quantity and quality of sperm was decreased in the months leading to comparatively higher sperm production in NBS.

However, in a study Blottner *et. al.* (2005) concluded that the number of motile sperm declined to 15 and 18 % in May and December (range 5 – 40 %), and of morphologically intact sperm to 51 % in both seasons further cryopreservation of sperm during December results in survival rates similar to those measured during the breeding season, even more important for successful preservation is the selection of suitable semen donors.

One reason for seasonal differences in the structure and functional integrity of plasma membrane could arise from the differences in the composition and hormonal content of the seminal plasma (Hoffmann and Landeck, 1999). Factors of seminal plasma show interactions with the sperm surface and modify membrane characteristics. Another possible cause is the difference in the efficacy of spermatogenesis. Present finding is contrary to study (Johnson, 1991) who postulated that sperm and testosterone production show seasonal fluctuations and sub optimal conditions during the non breeding season could result in changes of sperm quality. Different cellular structures of stallion spermatozoa have different susceptibilities to sub optimal conditions or physiochemical stress.

Maximum of previous studies reported in literature have been carried out in cold climate countries, where there is no summer stress and hence there is better seminal attributes in BS in their study which is in contrast to our study. Various other workers have supported the view that maintenance

of normal function of sperm membrane is a crucial pre requisite for sperm viability as well as reactivity at the site of fertilization. Increased proportion of spermatozoa with damaged membranes is indicative of reduced fertility (Curry *et. al.*, 1995).

It can be concluded that all the seminal attributes studied were significantly better in NBS as compared to BS. So freezing semen during NBS gave better post thaw values than during BS. An overall appraisal of the study on the use of different extenders revealed that extender E4 was considered best as it preserves maximum per cent motility, viability and positive sperms to HOS in stallion semen, so combination of cryoprotectant worked better as compared to single cryoprotectant. Cryoprotectant like DMF and EG preserve stallion semen in an almost similar way, however, GLY was found an inferior cryoprotectant.

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