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Study to Evaluate the Time Dependent Changes in Frozen-Thaw Goat Spermatozoa Prior to Insemination

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ABSTRACT

A study was conducted to evaluate the time dependent post thaw semen quality of goat during the holding period prior to insemination. Frozen semen straws from 10 healthy Barbari buck maintained at frozen semen production station were utilized for the experiment. Frozen semen dose of each individual buck was subjected to thawing later kept at room temperature. The seminal attributes were recorded at every 5 minutes interval starting from 0 minute up to 30 minutes using computer assisted semen analyzer (CASA) and flow cytometer. A significantly ($p \leq 0.01$) higher values for percent viable, acrosomal intact, polarize spermatozoa indicative of mitochondrial function along with reactive oxygen species (ROS) unaffected spermatozoa were observed up to 5 minutes of post thawing. The value exhibited a significant ($p \leq 0.05$) increase curvilinear velocity (VCL, µm/sec), average path velocity (VAP, µm/sec), straight line velocity (VSL, µm/sec), Linearity (Lin%), Straightness (Str %), Wobble (WOB%), beat cross frequency (BCF %) and maximum amplitude-lateral head displacement (ALH, µm) at 5 minutes and 10 minutes of thawing which significantly ($p \leq 0.05$) decreased with increase in holding time after thawing. So, it may be concluded that frozen thaw spermatozoa retrieve the metabolic activity within 5 minutes of thawing thereafter exhibit reduction in seminal attribute, hence insemination within 5 minutes of thawing can be considered as the best suited time to utilize semen for insemination with better conception.

HIGHLIGHTS

- Goat spermatozoa require 5 minutes to retrieve its metabolic activity and there after starts exhibits detrimental changes.
- Artificial insemination should be conducted within 5 minutes post thawing.

Keywords: Artificial insemination, goat, semen, post thaw semen quality, thawing

Spermatozoa is the basis life. It is a highly motile male gamete designed to carry and transfer the genetic material from male to female reproductive tract for fertilization (Sakkas *et al.,* 2015). During natural mating, sperm are directly transferred from the male reproductive tract into the female reproductive tract without any exposure to external environment. Sperm are strictly maintained at a temperature of 37°C throughout their entire journey staring from spermatogenesis, storage, insemination in female reproductive tract up to fertilization (Çok *et al.,* 2015).

Today, artificial insemination has come up as an assisted reproductive technique for wider dissemination of superior germplasm. Artificial insemination preferably utilizes cryopreserved semen that involves semen collection through artificial means (using artificial vagina, electroejaculation) its dilution with semen extender, equilibration, freezing and finally thawing of frozen semen for its use as insemination dose (Saha *et al.,* 2022). During the process of semen cryopreservation sperm cells

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are exposed to variable range of temperature ranging from 37°C to -196° involving 5 hours for complete processing. Initial hours of freezing are considered to be curtail for the sperm health (Palomar *et al.,* 2018). Subjecting metabolically active spermatozoa to variable temperature range in short duration induce stress resulting in metabolic dysfunction and overproduction of ROS that affect the sperm plasma membrane making sperm liable to freeze-thaw injuries.

Artificial insemination in goat is new techniques recently introduce in Indian animal husbandry system. A little has been studied about the behavior and response of spermatozoa during semen cryopreservation and artificial insemination in goat. Presence of bulbourethral enzymes (phospholipase A_2) make the process of semen cryopreservation and post thaw spermatozoa survival difficult in goat (Roy., 1957). Increased susceptibility to variable temperature and higher sensitivity of goat sperm in semen diluter requires a better understanding of time dependent changes to judiciously use insemination dose for higher conception. Majority of research have focused on effect of minerals supplementation on animal health and semen quality (Kesari *et al.,* 2022; Singh *et al.,* 2019), membrane protectants (Anand *et al.,* 2017) and ROS regulators in semen diluter, but to best of our knowledge on study has been conducted to evaluate the threshold time to hold the frozen thaw semen dose prior to insemination to improve the conception rate. So, to determine ideal holding time of the frozen thaw semen dose prior to insemination, the study was designed to investigate the time dependent changes in the post thaw semen prior to insemination.

MATERIALS AND METHODS

The study was conducted at semen evaluation and certification laboratory, department of veterinary physiology, DUVASU, Mathura. Ten frozen semen doses from different breeding buck were collected from the goat frozen semen production station DUVASU. The semen doses were thawed individually in vertical thawing unit maintained at 37°C for 40 second. The thaw semen was transferred to 1.5 ml plastic tube and kept for experimentation at room temperature. To evaluate the seminal attributes 10µl of semen was taken and mixed with 90 µl of diluter at 37°C to reach the final concentration of 40 million spermatozoa per ml as sample stock solution. To evaluate the effect of thawing practices on sperm kinematic and path velocities, 10 µl of stock solution was placed on 8 chambered leza slide for even distribution of sperm cell within the field. The slide was placed on the warm stage and evaluated under phase contrast with 10x objective using CASA with specific software. A total of five fields were randomly evaluated through automated selection. To evaluate the viability, acrosomal integrity, ROS affected spermatozoa and membrane fluidity, the easy kits were utilized for assessment under flow cytometer and experiment was performed as per the procedure described. The process was repeated for each individual sample at an interval of 5 minute, from 0 to 30 minutes.

STATISTICAL ANALYSIS

Statistical analysis was performed using Statistical Package for Social Science (SPSS® Version 20.0 for Windows®, SPSS Inc., Chicago, USA). The means were compared using Analysis of Variance and presented as mean ± standard error (SE).

RESULTS AND DISCUSSION

Time dependent changes in the sperm characters were studied in the post thaw semen stored at room temperature. During the experiment, the result recorded for % spermatozoa is affected with (ROS) were non-significantly lower from 0 to 5 minutes which later increase significantly ($p \leq 0.01$) with increase in holding time from 10 minutes and 30 minutes (Fig. 1).

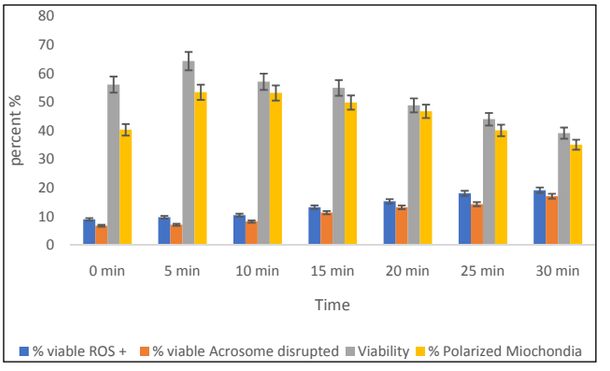

Fig. 1: Seminal attributes exhibited by frozen thawed spermatozoa at different time during storage at room temperature

Subjecting the metabolically active spermatozoa outside the reproductive tract (at room temperature) induce stress

Table 1: Motion kinematics exhibited by frozen thawed spermatozoa at different time during storage at room temperature

Parameter \ Time	0 min	5min	10 min	15 min	20 min	25 min	30 min
% Motile	51.00 ±2.98^{ab}	57.00 ±1.30^{b}	50.40 ±4.32^{ab}	47.80 ±4.18^{ab}	44.00 ±2.90^{ab}	41.60 ±1.86^{a}	37.40 ±1.48^{a}
% Progressive	32.20 ±1.59^{cd}	35.20 ±1.35^{d}	30.80 ±2.08^{bcd}	25.20 ±2.43^{abcd}	25.60 ±2.29^{abc}	23.00 ±2.38^{ab}	20.40 ±1.08^{a}
VCL (µm/sec)	100.20 ±8.48^{ab}	116.60 ±5.40^{b}	100.00 ±8.85^{ab}	92.80 ±11.31^{ab}	84.00 ±8.90^{ab}	76.60 ±6.40^{a}	70.20 ±6.27^{a}
VAP (µm/sec)	59.40 ±4.95^{bc}	69.80 ±2.39^{c}	58.00 ±4.52^{bc}	51.00 ±5.75^{abc}	46.60 ±4.79^{ab}	42.20 ±3.56^{ab}	38.00 ±3.43^{a}
VSL (µm/sec)	49.40 ±3.90^{bc}	57.60 ±2.06^{c}	48.00 ±3.78^{bc}	48.80 ±4.35^{ab}	38.20 ±3.87^{ab}	34.20 ±3.13^{ab}	30.20±2.72^{a}
STR%	41.00 ±2.16^{bc}	45.80 ±1.20^{c}	40.40 ±2.24^{bc}	37.20±3.29^{abc}	34.80 ±3.39^{abc}	32.60 ±2.50^{ab}	29.00 ±1.58^{a}
Lin%	25.20 ±1.35^{bc}	28.40 ±1.03^{c}	23.80 ±1.46^{bc}	21.00±1.78^{ab}	20.20 ±2.02^{ab}	18.40 ±1.72^{ab}	16.00±1.22^{a}
ALH (µm)	4.15 ±0.37^{ab}	5.20 ±0.20^{b}	4.40 ±0.40^{ab}	4.21±0.49^{ab}	3.80 ±0.37^{ab}	3.60 ±0.40^{ab}	3.20 ±0.20^{a}
BCF%	20.00 ±1.00^{ab}	22.40 ±1.03^{b}	19.80 ±1.53^{ab}	17.40 ±1.80^{ab}	16.00 ±1.64^{a}	15.11±1.18^{a}	14.13±1.22^{a}

Mean values marked with the capital letter show difference at ($P \leq 0.01$); Different superscripts (a, b,c,d) with in rows differ significantly.

(Rahman *et al.,* 2018). Stress disturbs the mitochondrial energy production system in the spermatozoa leading to higher production of ROS (Chianese and Pierantoni., 2021). Although the seminal fluid has sufficient antioxidative activity to control the ROS production but overproduction make the seminal antioxidant insufficient to counter balance the ROS generated by spermatozoa is reflected through altered sperm characters. The ROS concentration in seminal plasma also has a positive correlation with dead and damaged spermatozoa in medium (Takeshima *et al.,* 2020). During the experiment, a non-significant difference in values recorded at 0 and 5 minutes may be attribute to the time for retrieval of sperm metabolic activity that delayed the production of ROS from spermatozoa. Mitochondria is the major site of energy production as well as ROS generation. Thawing process induce stress on sperm cell alter the electron transport change in mitochondrial cistern leading to ROS production (Nowicka-Bauer and Nixon., 2020). During the experiment significantly ($p \leq 0.01$) lower values of polarized sperm indicative of metabolically active sperm were observed at 0 min that increase significantly from 5 minutes and again decreased significantly after 20 minutes of thawing. The lower values for % ROS +ve sperm from 0 to 5 minutes may be attributed to delayed ROS production during metabolic revival after thawing. Significantly ($p \leq 0.01$) higher values thereafter may be the result of increase in ROS production by the metabolic active spermatozoa undergoing thermal stress of freeze thawing. The degree of acrosomal damage in the sperm cell exhibited the similar patter with significantly ($p \leq 0.01$) lower values at 0 and 5 minute of thawing which increase significantly after from 15 minute to 30-minute post thawing (Fig. 1). Acrosomal integrity is the function of plasma membrane. An intact membrane maintains the integrity of acrosome preventing capacitive changes and acrosomal reaction prior to release of sperm in the female reproductive tract. ROS act upon the plasma membrane of sperm cell leading to efflux of cholesterol and phospholipids (Sheriff *et al.,* 2010). Limited production of ROS prepares the spermatozoa for important fertility processes that includes capacitation and acrosomal reaction. But over production of ROS leads to acrosomal damage, release of intracellular calcium and induce pre mature capacitation and acrosomal reaction (Qamar *et al.,* 2023). During the experiment, the higher values of ROS affected sperm after 5 of thawing when compared with results for acrosomal damage indicates ROS induced membrane damage resulting in acrosomal disruption. Significant difference in the values for viability was evident at 20 minutes post freeing (Fig. 1). The lower values of percent viable spermatozoa resulted from the increased ROS level, causing plasma membrane destabilization leading to loss of selective permeability of membrane that disturbed in the sperm internal environment and energy production system loss of viability and mitochondrial trans-membrane potential.

The values recorded for motility, path velocity and kinematic characters has been presented in table 1. During the experiment the total motile spermatozoa and spermatozoa exhibiting progressive movement together

with the kinematic *viz.* curvilinear velocity (VCL, µm/sec), average path velocity (VAP, µm/sec), straight line velocity (VSL, µm/sec), Linearity (Lin%), Straightness (Str %), Wobble (WOB%), beat cross frequency (BCF %) and maximum amplitude-lateral head displacement (ALH, µm) exhibited significantly higher values from 0 to 5 minutes that decrease significantly thereafter from 10 minutes to 30 minutes. Sperm motility is the sensitive to any change in the environment round the sperm. Motility is the coordinated function of spermatozoa that involves flagellar machinery, energy production mechanism, its transfer and utilization by flagellar tail for forward progression (Anand *et al.,* 2017). Glucose is the main source of energy for goat spermatozoa. Spermatozoa derives its energy from the medium in which it is suspended. Without external energy source, spermatozoa are limited to available energy in diluted seminal plasma. Constant use of energy for motility not only depletes available energy source and reduced capacity of flagellar machinery to utilize the energy (Anand *et al.,* 2018). The frozen thaw spermatozoa have a higher tendency to produce ROS. Spermatozoa membrane also becomes more liable to membrane damage during the process of freezing and thawing (Khan *et al.,* 2021). During the experiment depletion of energy source, increase ros production by spermatozoa in medium together with the susceptible plasma membrane of sperm cell led to sperm membrane damage leading to change in sperm internal environment affecting energy production and its transfer to tail. Since the extent of damage increase with time, the evident decrease in motility with increase in time may be the result of ROS and mitochondrial dysfunction.

So, it can be concluded that frozen thaw sample exhibits detrimental changes in spermatozoa after 5 minutes of thawing. Kinematic character is first followed by ROS damage and acrosomal disruption leading to pre-mature capacitation and later loss of viability. So, it may be concluded that frozen thaw goat semen sample should be ideally utilized for insemination within 5 minutes after thawing.

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