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EVALUATE THE EFFECT OF EGG YOLK AND SEMINAL PLASMA ON SPERMATOZOA ABNORMALITY USING DIFFERENT CRYOPRESERVATION PROTOCOL IN BARBARI BUCK SEMEN

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Abstract: The experiment was conducted to assess the effect of egg yolk and seminal palsma on spermatozoa abnormality using different cryopreservation protocol in Barbari buck. Five Barbari bucks aged between 2-4 years, weighing 25-35 kg were selected as semen donor. Semen was collected twice a week from each buck and a total of 30 ejaculates (6 ejaculates per buck) were used in this study. After initial evaluation, each semen samples were pooled and split into three groups *viz.* T-1 (3% egg yolk), T-2 (20% egg yolk) and T-3 (20% egg yolk after sperm washing) with final concentration of 100×10^6/ml. Percent abnormal spermatozoa were evaluated at each step i.e. just after dilution, after equilibration and freez-thawing during the processing. A significant difference ($P \leq 0.01$) was observed in the percent abnormal sperm at different steps of freezing, both within and between the treatment groups. The result indicates that the values recorded for percent abnormal spermatozoa were significantly ($P \leq 0.01$) lower in T-1 just after dilution while higher ($P \leq 0.01$) values were recorded in T-3 after equilibration and in T-1 after freez-thawing. So it may be concluded that the factor affecting the percent abnormal sperms during the cryopreservation vary at different stages of processing, ranging from lethal interaction between egg yolk and bulbourethral secretions at dilution to seminal plasma during equilibration and egg yolk during deep freezing and thawing.
Keywords: Barbari buck, cryopreservation, egg yolk, seminal plasma.

Introduction

Semen cryopreservation is the process to preserve spermatozoa at ultra low temperature for prolonged period of time. During this process, the spermatozoa are subject to variable temperature that makes them susceptible to cold shock and cryoinjury. Temperature variations and cell dehydration induce changes in lateral phase separation of lipids, resulting in lateral reordering of membrane components (Drobnis et al, 1993) and the loss of poly-unsaturated fatty acids and cholesterol (Maldjian et al, 2005; Chakrabarty et al, 2007). This alters the permeability of the sperm surface to water, ions and cryoprotectants (Hagiwara et

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al, 2009; Oldenhof et al, 2010) affecting the plasma membranes over the principal-piece, midpiece, and head leading to increase in percent abnormal spermatozoa (Holt and North, 1994) that inturn affect fertility.

Egg yolk is used as a common cryoprotectant in semen extenders for domestic animals. However, the dilution of goat semen with extenders containing egg yolk or milk has a detrimental effect on the semen quality during freezing and thawing. Presence of egg yolk-coagulating enzyme (EYCE) and bulbourethral gland secretion glycoprotein (BUSgp60) (Pellicer-Rubio and Combarnous, 1998; Sias et al, 2005) results in a lethal interaction between egg yolk and seminal plasma affecting morphology and physiology of spermatozoa. Considering this fact different protocols *viz* 3% egg yolk, 20% egg yolk at lower spermatozoa concentration and 20% egg yolk after sperm washing have been proposed for cryopreservation of goat semen, giving an impression that egg yolk and seminal plasma are most important factor determining semen quality during freezing thawing process. So, taking into account these facts present experiment was conducted to evaluate the role of egg yolk and seminal plasma on percent abnormal spermatozoa using different cryopreservation protocol at different steps involved during freezing thawing process.

Materials and Methods

Five normal, healthy adult Barbari bucks aged between 2- 4 years, weighing 25-35 kg reared at the experimental goat sheds of Department of Physiology were used as semen donor. Semen was collected twice a week using artificial vagina. A non-estrous doe was used for mounting of bucks and semen was collected. Samples with more that 85% live spermatozoa were selected and later pooled. The pooled semen sample was divided into three equal parts, later diluted in the semen extender (TRIS) according to the treatments - T1-3% egg yolk or T2-20% egg yolk. For T-3 treatment, the semen was also washed before cryopreservation and diluted with extender containing 20% egg yolk and cryopreserved. Percent morphological abnormalities in a semen sample were evaluated using eosin-nigrosin staining technique (Hancock, 1952) in all the three treatment groups. At least 200 spermatozoa were counted under the oil immersion objective (100 X) under phase contrast microscope and percent abnormal spermatozoa were calculated. The result were analyzed using a two-way analysis of variance, followed by a Tukey's post hoc test to determine the significant differences both within and between different treatment groups, using the SPSS/PC computer program (version 14.0; SPSS, Chicago, IL, USA).

Results and Discussion

The mean (±SE) values of percent abnormal spermatozoa for 6 ejaculates of buck no.1, 2, 3, 4 and 5 were 3.50±0.34, 3.83±0.31, 3.83±0.54, 4.33±0.33 and 4.83±0.48 with a mean (±SE) values of 4.07±0.19, respectively. A non-significant difference (P≥0.05) was observed in the semen samples collected, hence samples were pooled. The mean (±SE) of percent abnormal sperms observed in different treatment groups has been presented in Table-1. Many structural abnormalities of spermatozoa may be due to faulty spermatogenesis, diseases and adverse environmental conditions to which sperms are subjected. During the process of semen cryopreservation the sperm cell are subject to variable conditions that includes freezing medium, the cryoprotectant (CPA) and its concentration, the freezing conditions and cooling and warming temperatures which influence post-thawing sperm motility and membrane integrity, leading to increased proportion of abnormal sperm (Moce and Vicente, 2010). Available literature suggests that the increase in morphological abnormality deteriorates the semen quality and becomes inadmissible above 20 per cent (Hafez, 1993). During the experiment the percent abnormal sperms were well with the admissible limit but increased significantly at each subsequent step involved in process of cryopreservation. The observed mean (±SE) values of percent abnormal spermatozoa in semen just after dilution in T-1 (0.3% egg yolk without centrifugation), T-2 (20% egg yolk without centrifugation) and T-3 (20% egg yolk with centrifugation) were 5.13±0.20, 5.07±0.20 and 6.73±0.22, respectively. The observed mean (±SE) values of percent abnormal spermatozoa in semen after equilibration were 6.07±0.15, 5.67±0.15 and 8.03±0.15 and in freeze-thawed semen were 9.80±0.37, 6.83±0.24 and 8.80±0.20, respectively. A significant difference (P≤0.01) was observed both within and between the three trial groups selected during the experiment. Lowest values observed in the diluted semen and highest at post thaw in the three trial group, indicating a adverse effect of cooling on the percent abnormal spermatozoa. Connell et al. (2002) observed a significant increase in number of abnormal sperms during freez-thawing. Significantly higher (P≤0.01) value of percent abnormal spermatozoa was observed in the T-2 followed by T-1 and T-3 just after dilution. This may be the result of lethal interaction between the egg yolk and seminal plasma (Roy, 1957) that led to increase of abnormal spermatozoa. The observed mean± SE values for percent abnormal spermatozoa were highest in the T-3 followed by T-2 and T-1 after equilibration. The reason for this difference may be reduction in temperature during equilibration that stimulated production of ROS. Seminal plasma has antioxidative enzymes that regulate the overproduction of ROS during the

lowering of temperature (Shi et al, 2010). This antioxidant system comprising GSH, GPx, Catalase (CAT) and SOD act as a defense functioning mechanism against the Lipid Peroxidation (LPO) of semen, maintaining sperm quality (Agrawal et al, 2007). The results observed in the semen samples after equilibration when compared with the after dilution indicates the role of seminal plasma through its antioxidative enzymes in preventing sperm abnormality, resulting in higher values of percent abnormal sperms in T-3 with seminal plasma removed through washing. The values observed in the post thaw semen sample were the highest percent of abnormal sperm in the T-1 followed by T-3 and T-2. The result indicate that egg yolk concentration which was higher in T-2 and T-3 compared to T-1 may be the major determining factor through its cryoprotective action to overcome freez-thawing stress primary to seminal plasma, preventing cell distortion. So it may be concluded that factor regulating the percent abnormal sperms during the cryopreservation process vary at different stages of semen processing ranging from lethal interaction between egg yolk and bulbourethral secretions at dilution to seminal plasma till equilibration and egg yolk during deep freezing and thawing. Further studies need to be conducted to study the interactive mechanism of seminal plasma and egg yolk with sperm cells membrane at variable temperature during cryopreservation steps to improve freezing thawing process for better post thaw semen quality.

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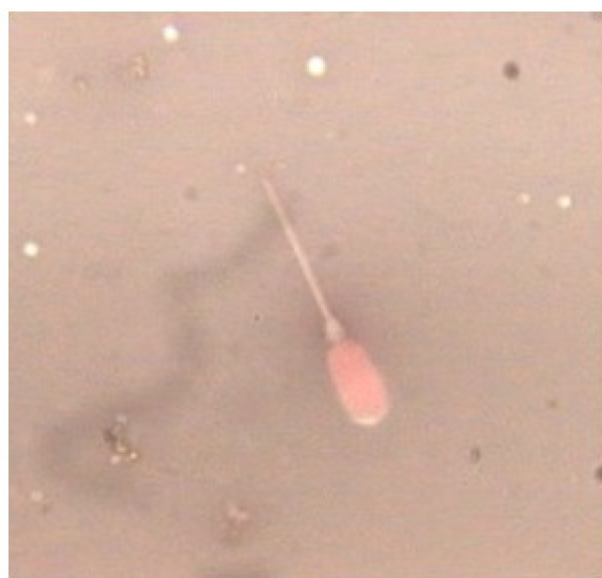
Table 1. Percent abnormal spermatozoa during the different stages of processing and addition of different levels of egg yolk in Barbari buck spermatozoa

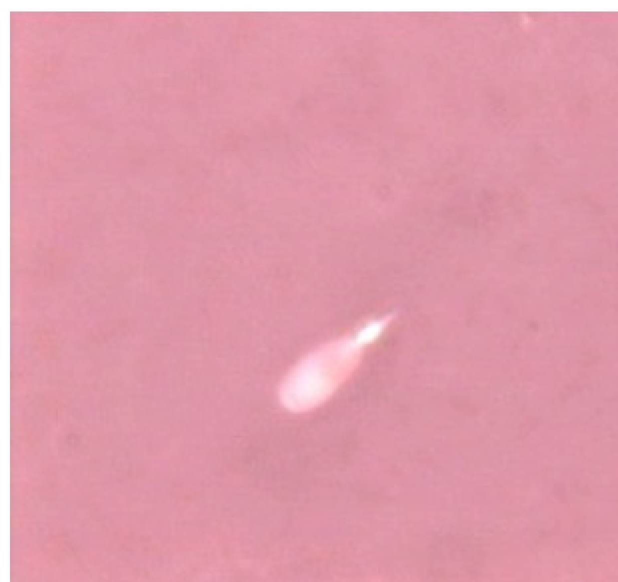
Stages of processing	**Percent Abnormal Spermatozoa**		
Level of egg yolk in extender	**03 % egg yolk in semen extender (group I)**	**20 % egg yolk in semen extender (group II)**	**20 % egg yolk in semen extender after sperm washing (group III)**
Just after dilution	5.13 ± 0.20^{Ab} (30)	5.07 ± 0.20^{Aa} (30)	6.73 ± 0.22^{Ac} (30)
After equilibration	6.07 ± 0.15^{Bb} (30)	5.67 ± 0.15^{Ba} (30)	8.03 ± 0.15^{Bc} (30)
After thawing	9.80 ± 0.37^{Cb} (30)	6.83 ± 0.24^{Ca} (30)	8.80 ± 0.20^{Cc} (30)

Figure in parenthesis indicate number of observations.
Different superscript for value in rows and columns are significantly different (P≤0.01).

Figure 1- Photograph showing spermatozoa with abnormalities (Eosin-Nigrosin stain, Magnification: 100x)



(Cytoplasmic droplet)



(Decapitated head)