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Research Article

THE EFFECT OF PLATELET-RICH-PLASMA SUPPLEMENTED EXTENDER ON POST-THAW FERTILITY OF SIROHI AND BARBARI BUCK SEMEN

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ABSTRACT: The freezing and thawing process during cryopreservation of semen decreases motility, compromises membrane integrity and impairs cell function, along with the fertilizing capacity of the spermatozoa. The present study aimed to evaluate the efficacy of platelet-rich plasma (PRP) as a supplement in semen extenders to improve post-thaw fertility of Barbari and Sirohi buck semen. For this study, 288 ejaculates were collected from both breeds, of which 30 best ejaculates from each breed were processed and cryopreserved using a standard protocol. Each ejaculate was divided into three equal aliquots. A tris egg-yolk citrate extender was utilized for dilution, to which varying percentages of PRP were incorporated. The first aliquot of semen was diluted with extender containing 0% PRP (G-I), the second with extender containing 7% PRP (G-II) and the third with extender containing 10% PRP (G-III), respectively. All the ejaculates were evaluated at pre-freeze, equilibration and post-thaw stages. At the pre-freeze stage, semen parameters did not vary significantly across different extenders. However, significantly higher ($p<0.05$) percentage of progressive motility (54.00±1.54,53.66±1.49), sperm viability (62.73±1.54,58.36±1.44) and plasma membrane integrity (51.63±1.85,50.23±2.43) was recorded during post post-thaw stage in Barbari and Sirohi bucks extender supplemented with 7 % PRP. The inclusion of 7% PRP enhanced post-thaw fertility and had a beneficial impact on the semen freezability of Barbari and Sirohi bucks.

Keywords: Cryopreservation, Platelet-rich plasma [PRP], Buck semen, Sirohi, Barbari.

INTRODUCTION

Cryopreservation of semen is essential for maintaining sperm motility and viability for extended periods. However, it is a complex process that can damage the various aspects of the cell membrane along with its function, ultimately diminishing its overall functionality. Cryoinjuries primarily affect sperm motility, plasma membrane functionality and acrosome integrity [1]. Platelet–Rich Plasma (PRP) is a substance, abundant in growth factors that are compatible, homogeneous and non-antigenic. Platelets contain α granules, which on activation release seven fundamental protein growth factors, viz. epidermal growth factor, insulin-like growth factor, vascular endothelial growth factor, transforming growth factor β, nerve growth factor, hepatocyte growth factor and fibroblast growth factor, respectively [2]. Platelets also contain dense platelet granules consisting of histamine, zinc ions, calcium ions, superoxide dismutase, serotonin and adenosine triphosphate, all of which play a crucial role in maintaining cellular and tissue homeostasis [3].

Numerous preventive procedures are implemented to minimize the detrimental effects of semen cryopreservation, including optimizing freezing protocols, engineering freezing apparatus and improving the freezing medium [4]. The addition of cryoprotectants has become the most scientifically researched method, along with antioxidants, peptide molecules, fatty acids, animal serum, nanoparticles, plant essential oils and bioactive analogues. This has proven beneficial for improving sperm quality and viability after cryopreservation. The formulation of the semen extender used in cryopreservation plays a vital role in preserving the integrity of spermatozoa [5].

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Supplementation of PRP in semen extender at various concentrations has been validated to enhance sperm quality in both fresh and post-thawed conditions in ram at 5% PRP [6], buck at 10% PRP [7], buffalo bull at 10% PRP [8] and human at 5% PRP [5]. The growth factors present in PRP protect the sperm from cryodamage during cryopreservation, which helps maintain sperm viability, acrosomal integrity, plasma membrane integrity and fertility. It also improves both *in vivo* and *in vitro* fertilization, making it a highly effective technique for enhancing fertility [7, 8]. The present study aims to investigate the effectiveness of PRP in preserving sperm fertility during cryopreservation by examining its impact on various physiological parameters. No literature could be traced regarding the potential effects of incorporating Platelet-Rich Plasma (PRP) concentrations into a semen extender on sperm quality and functionality in Indian goat breeds. Therefore, this study was formulated to evaluate the post-thaw fertility of PRP-supplemented buck semen and its effect on the post-thaw recovery rate.

MATERIALS AND METHODS

Animals and semen collection

Six sexually mature adult bucks of each Barbari and Sirohi breeds were selected for this study. The bucks were maintained under a semi-intensive system in the University Goat Farm, NDVSU, Jabalpur, Madhya Pradesh. For six months, routine semen samples were collected with the help of an artificial vagina and rapid testing was done at the field level for assessment of freezability. Ejaculates fulfilling minimum standards were selected for further dilution and then cryopreservation.

PRP preparation

The PRP was prepared by the modified double centrifugation protocol adopted from Salama and co-workers [7]. Briefly, 3ml of whole blood was collected in anticoagulant acid citrate dextrose-A (ACD-A) coated tubes from four additional, sexually mature bucks of both breeds on the day of semen collection and centrifuged initially at 2450 rpm for 5 mins. The supernatant and buffy coat were transferred to another sterile anticoagulant-free tube and re-centrifuged at 3300 rpm for 17 mins to obtain pure PRP. The manual smear slide method was used to calculate platelets in PRP, and a concentration of 10^9 cells/ml PRP was maintained [9]. PRP activation was done by adding 10% calcium chloride in a 1:10 ratio, and the supernatant was used for this study.

Ethical Consideration

The Institutional Animal Ethical Committee of the College of Veterinary Science and Animal Husbandry, Jabalpur, Madhya Pradesh, approved all the animal experiments conducted in this study (certificate no: 13/IAEC/Vety./2024)

Extender preparation

A tris-based semen extender was prepared using tris, citric acid, fructose, 6% glycerol, 10% egg yolk, streptomycin and penicillin dissolved in double-distilled water to make 100ml volume as standardized in the Semen processing lab of the Department of Veterinary Gynaecology and Obstetrics, Jabalpur. The pH was adjusted to 7.0 to 7.2. PRP was added in concentrations of 7%(G-II) and 10%(G-III) to 100 ml of semen extender; the control (G-I) was devoid of PRP.

Semen handling and cryopreservation

The freshly collected semen ejaculates were divided into three equal aliquots. The initial dilution of 1:3 was performed at the farm upon evaluation, followed by a final dilution depending on the sperm concentration in the lab at 4°C before cryopreservation. Post dilution, followed by 4 hours of equilibration, the filling and sealing of semen straws was done. After that, straws were positioned on a freezing rack, 4 cm above the static liquid nitrogen vapor. After seeding for 10 minutes, the straws were immediately plunged into liquid nitrogen and stored at -196°C until further evaluation. A total of 288 ejaculates were collected from both breeds throughout the study interval, out of which the best 60 ejaculates (30 of each breed) were used to assess the effect of PRP as a semen extender at different semen processing stages. Post-thaw semen assessment was done after thawing at 40ºC for 45 sec.

Evaluation of semen

The collected semen samples were evaluated for sperm individual motility, viability, plasma membrane integrity, abnormalities and acrosomal integrity at three stages of semen processing, i.e., pre-freeze, equilibrated and post-thaw stage.

Individual motility (%)

Initial individual motility was evaluated by placing a 10µl drop of diluted semen on a sterilized pre-warmed (37 °C), clean, grease-free glass slide. It was observed under the high-power objective lens (400X) of the compound phase contrast microscope. The percentage of motile spermatozoa was calculated subjectively by

observing the direction and movement of each sperm. Further processing was done on ejaculates that exhibited at least 70% motility [10].

Sperm Viability (%)

The sperm viability (%) was evaluated using the method described by Blom [11]. A small drop of diluted semen was mixed with an equal amount of Eosin-Nigrosin stain in a 1:4 ratio, and a thin, uniform tongue-shaped smear was prepared. After drying, a total of 200 sperms were counted in various fields under a high-power objective lens (400X) of the compound phase contrast microscope. Unstained spermatozoa heads were considered live and partially /completely stained as dead [Figure 1a].

Sperm plasma membrane integrity (%)

The evaluation of sperm plasma membrane integrity was done by the hypo-osmotic swelling (HOS) test as per the method described by Revell and Mrode [12]. Briefly, 0.1ml of diluted semen sample was mixed with 1ml of HOS solution and incubated in a water bath at 37°C for 1 hour. Post incubation, a 10µl drop of sperm suspension was placed on a clean, grease-free glass slide and covered with a coverslip. Two hundred sperms were examined in various fields under a high-power objective lens (400X) of the compound phase contrast microscope for sperm tail swelling. Spermatozoa showing any kind of swelling or curling of the tail were considered as HOST-reacted sperm [Figure 1b] and expressed in percentage. The osmolarity of the HOS solution differed during the pre-freeze (150mOsmol/L) and post-thaw stages (75mOsmol/L) as per the method adopted by Gangwar and co-workers [10].

Acrosomal integrity (%)

The acrosomal integrity percentage was assessed using the Giemsa staining technique as per the method given by Gangwar and co-worker [10]. On a sterilized glass slide, a thin smear of each dilution was prepared and immediately fixed in methanol for thirty minutes after drying. The fixed semen smear was then stained for 90 minutes using freshly made Giemsa working solution (3ml Giemsa stock solution + 2ml Sorensen's phosphate buffer + 45ml distilled water). Spermatozoa having dark purple heads with visible acrosomal ridges were counted as live intact acrosomes, and spermatozoa with light purple/ white heads, remaining unstained or without an acrosomal cap, were counted as dead or non-intact spermatozoa [Figure 1c] and were expressed in percentage. 200 sperms were examined in various fields under a high-power objective lens (400X) of the compound phase contrast microscope.

Sperm abnormalities (%)

The morphological sperm abnormalities were studied using the Rose-Bengal staining technique [10]. A small drop of diluted semen was taken on a clean, grease-free glass slide and a thin, uniform smear was prepared. After drying, the slide was immersed in Rose-Bengal stain for 7 minutes. Following that, it was washed with tap water and air-dried. Two hundred sperm were examined in each smear at a magnification of 400X to determine the incidence of various sperm abnormalities classified as head, mid-piece and tail abnormalities [Figure 1d].

Statistical analysis

The data obtained during the study was subjected to statistical analysis with the help of the statistical software "R" Studio, Package. Data from different experiments were presented as Mean±SE and analyzed by Two-way ANOVA, and Tukey's range test as described by Snedecor and Cochran [13]. The difference at $p \leq 0.05$ was considered satisfactory significant.

RESULTS

Effect of supplementation of PRP in buck semen extender during different processing stages

Individual motility (%)

The individual motility percentage varied among groups across all stages, with a significant ($p < 0.05$) difference observed only at the post-thaw stage. The highest motility was recorded in 7% PRP-supplemented semen extender, followed by 10% and 0%, respectively [Tables 1 and 2].

Sperm viability (%)

For both breeds, sperm viability remained comparable during the pre-freeze and equilibration stages but showed a significant ($p < 0.05$) increase post-thaw in the 7% PRP-supplemented group [Tables 1 and 2].

Plasma membrane integrity (%)

Plasma membrane integrity was assessed by the proportion of HOS-positive spermatozoa and did not differ significantly between the pre-freeze and equilibration stages. However, significant difference ($p < 0.05$) was observed with the 7% PRP-supplemented semen extender, followed by 10% and 0%, as illustrated in Tables 1 and 2 in both breeds.

Acrosomal integrity (%)

The acrosomal integrity followed a similar trend and no significant ($p > 0.05$) difference was seen during pre-freeze and equilibration stage However, a higher value was seen in 7% PRP-supplemented semen extender, followed by 10% and then 0% in both breeds [Table 1 and 2].

Table 1. Seminal parameters in Sirohi buck semen supplemented with PRP at different stages (Mean±SE).

Parameters	Semen processing stages	Sirohi buck semen		
		0% PRP	7% PRP	10% PRP
Individual motility	**Pre-freeze**	$84.40^{a}\pm0.84$	$84.40^{a}\pm0.84$	$84.40^{a}\pm0.84$
	Equilibrated	$78.33^{a}\pm1.36$	$78.33^{a}\pm1.44$	$78.00^{a}\pm1.61$
	Post-thaw	$33.00^{bB}\pm1.92$	$53.66^{bA}\pm1.49$	$48.16^{bA}\pm2.19$
Viability	**Pre-freeze**	$87.96^{a}\pm0.90$	$87.96^{a}\pm0.90$	$87.96^{a}\pm0.90$
	Equilibrated	$83.86^{a}\pm1.14$	$86.30^{a}\pm0.96$	$82.63^{a}\pm1.40$
	Post-thaw	$41.86^{bB}\pm2.12$	$58.36^{bA}\pm1.44$	$51.96^{bA}\pm2.48$
HOS positive	**Pre-freeze**	$82.23^{a}\pm1.06$	$82.23^{a}\pm1.06$	$82.23^{a}\pm1.06$
	Equilibrated	$73.40^{a}\pm1.61$	$75.73^{a}\pm1.30$	$70.50^{a}\pm1.42$
	Post-thaw	$38.43^{bB}\pm2.39$	$50.23^{bA}\pm2.43$	$40.43^{bB}\pm2.40$
Acrosomal Integrity	**Pre-freeze**	$84.36^{a}\pm0.85$	$84.36^{a}\pm0.85$	$84.36^{a}\pm0.85$
	Equilibrated	$76.83^{a}\pm1.30$	$79.03^{a}\pm1.15$	$77.03^{a}\pm1.28$
	Post-thaw	$46.66^{bB}\pm2.21$	$62.93^{bA}\pm1.68$	$54.16^{bB}\pm2.42$
Abnormalities	**Pre-freeze**	$3.96^{c}\pm0.28$	$3.96^{c}\pm0.28$	$3.96^{c}\pm0.28$
	Equilibrated	$5.80^{b}\pm0.35$	$5.50^{a}\pm0.33$	$5.96^{b}\pm0.42$
	Post-thaw	$7.83^{aA}\pm0.43$	$6.16^{aB}\pm0.31$	$7.73^{aA}\pm0.47$

Mean value bearing different superscripts of the same parameter (a,b,c) within the group and (A,B,C) in between groups differ significantly ($p<0.05$).

Table 2. Seminal parameters in Barbari buck semen supplemented with PRP at different stages (Mean±SE).

Parameters	Semen processing stages	Barbari buck semen		
		0% PRP	7% PRP	10% PRP
Individual motility	**Pre-freeze**	$86.66^{a}\pm0.87$	$87.00^{a}\pm0.85$	$87.33^{a}\pm0.82$
	Equilibrated	$80.66^{a}\pm1.26$	$81.00^{a}\pm1.29$	$81.33^{a}\pm1.14$
	Post-thaw	$31.33^{bC}\pm2.29$	$54.00^{bA}\pm1.54$	$45.50^{bB}\pm2.27$
Viability	**Pre-freeze**	$90.83^{a}\pm0.64$	$90.83^{a}\pm0.64$	$90.80^{a}\pm0.63$
	Equilibrated	$87.16^{a}\pm0.93$	$87.13^{a}\pm0.93$	$86.76^{a}\pm0.74$
	Post-thaw	$39.50^{bB}\pm2.26$	$62.73^{bA}\pm1.54$	$56.00^{bA}\pm2.21$
HOS positive	**Pre-freeze**	$86.13^{a}\pm0.82$	$86.13^{a}\pm0.82$	$86.13^{a}\pm0.82$
	Equilibrated	$81.36^{a}\pm1.21$	$83.26^{a}\pm0.96$	$82.96^{a}\pm1.11$
	Post-thaw	$35.36^{bC}\pm2.27$	$51.63^{bA}\pm1.85$	$42.73^{bB}\pm2.36$
Acrosomal Integrity	**Pre-freeze**	$87.40^{a}\pm0.92$	$87.40^{a}\pm0.92$	$87.40^{a}\pm0.92$
	Equilibrated	$81.60^{a}\pm0.99$	$81.70^{a}\pm0.96$	$80.66^{a}\pm0.76$
	Post-thaw	$47.33^{bC}\pm2.25$	$68.90^{bA}\pm1.44$	$59.26^{bB}\pm2.15$
Abnormalities	**Pre-freeze**	$5.13^{b}\pm0.30$	$5.13^{b}\pm0.30$	$5.13^{b}\pm0.30$
	Equilibrated	$5.36^{b}\pm0.35$	$5.23^{a}\pm0.25$	$5.13^{b}\pm0.26$
	Post-thaw	$7.90^{aA}\pm0.32$	$5.76^{aB}\pm0.26$	$6.30^{aB}\pm0.33$

Mean value bearing different superscripts of the same parameter (a,b,c) within the group and (A,B,C) in between groups differ significantly ($p<0.05$) .

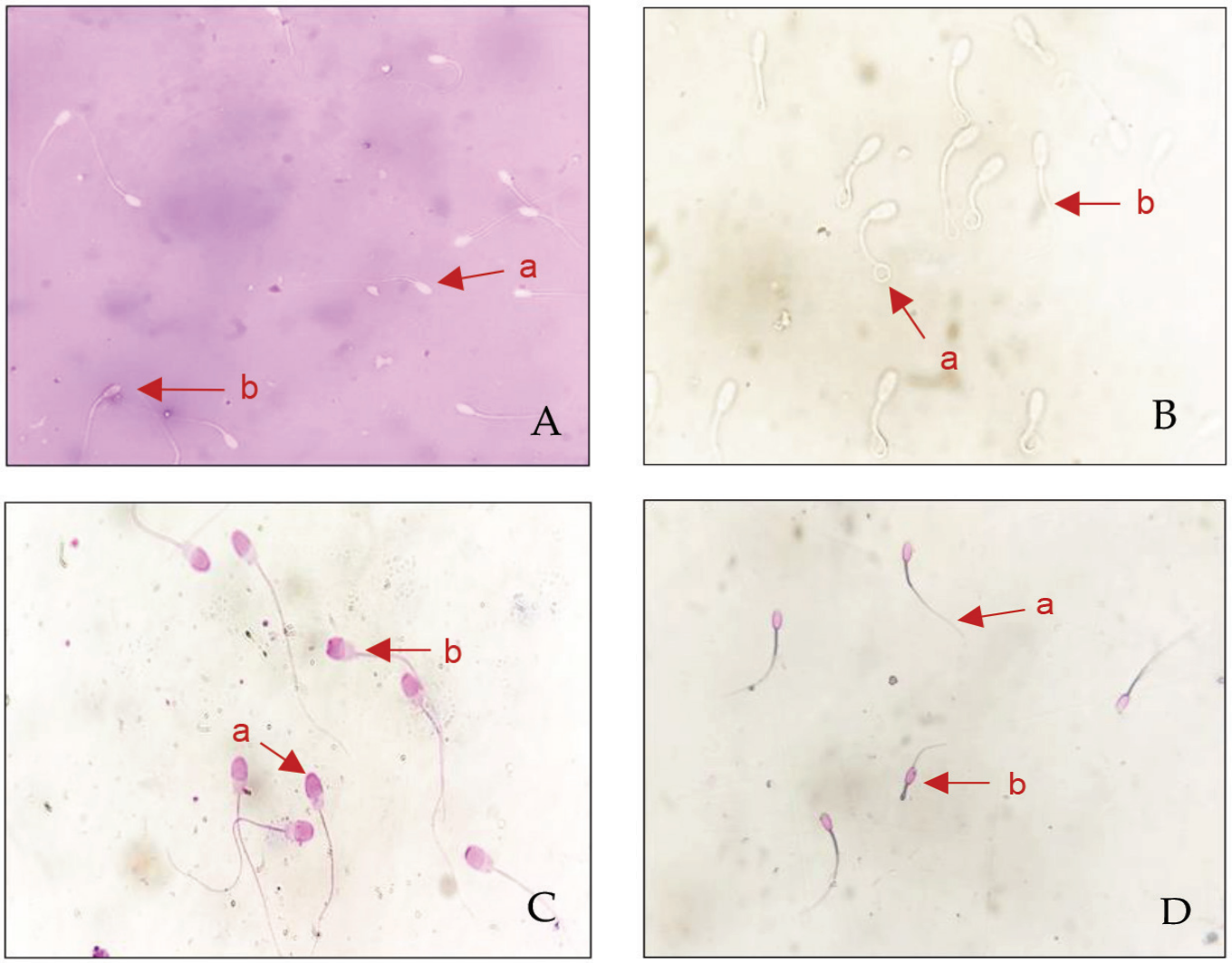

Fig. 1.A. Evaluation of viability of sperm. (a) Live – head colorless/white (b) Dead – pink/purple head. **B. Evaluation of plasma membrane integrity by HOST.** (a) Tail coiled - HOS reacted sperm (b) Tail no coiled - no reaction. **C. Evaluation of acrosome integrity of sperm.** (a) Acrosome intact (b) Acrosome reacted. **D. Evaluation of abnormalities.** (a) Abnormality absent (b) Abnormality present

Sperm abnormalities (%)

No significant ($p > 0.05$) differences were observed during early stages. Post-thaw, abnormalities were significantly ($p < 0.05$) lower in the 7% PRP group compared to 10% and control [Table 1 and 2].

Recovery rate percentage of semen parameters in PRP-supplemented semen extender of both breeds

The recovery rate percentage was calculated between the pre-freeze to post-thaw stage and the equilibrated to post-thaw stage, and the highest values of motility, viability, plasma membrane integrity, acrosomal integrity, and abnormality were found in the group supplemented with 7% PRP, followed by 10% PRP and then 0% PRP in both breeds (Figure 2).

DISCUSSION

The improvement in post-thaw sperm motility, viability, and membrane integrity may be attributed to the bioactive components of PRP, including insulin-like growth factor-1 (IGF-1), transforming growth factor (TGF), serotonin, and nerve growth factor (NGF). These molecules have been reported to enhance sperm function by improving calcium transport, reducing oxidative stress, and maintaining membrane stability. IGF-1 increases intracellular calcium levels, promoting flagellar activity and sperm survival [14], while TGF provides anti-inflammatory protection during cryopreservation [15]. Serotonin is known to improve sperm kinematics [16], and NGF enhances motility and viability by interacting with its receptor on the sperm mid-piece [17].

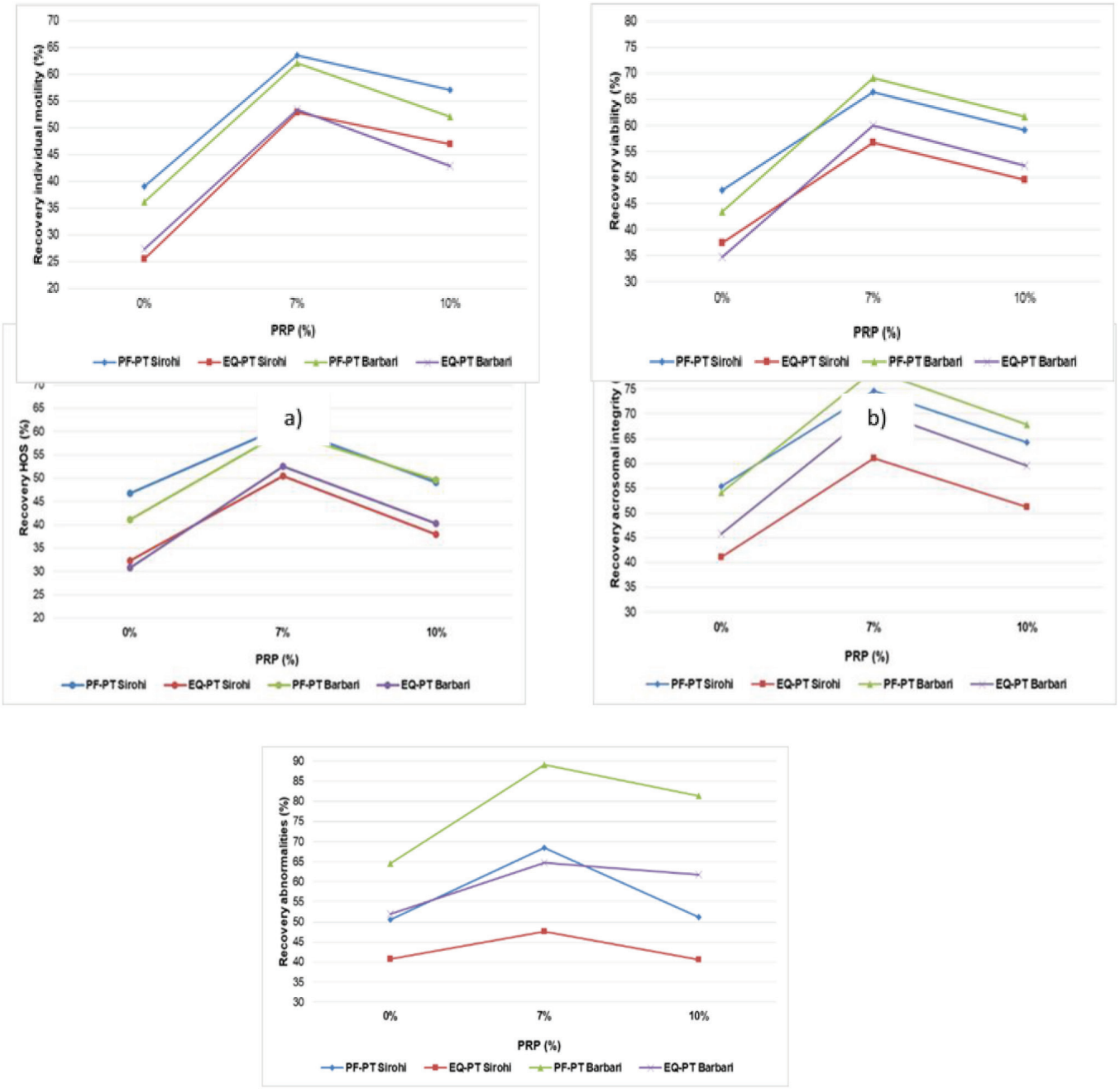

Fig. 2. Recovery percentage of spermatozoa in PRP-supplemented semen extender at different stages in both breeds. a) Individual motility, b) Sperm viability, c) Plasma membrane integrity by HOS, d) Acrosomal integrity and e) abnormalities. PF- pre-freeze, PT- post-thaw, EQ- equilibrated.

During freezing, spermatozoa are exposed to osmotic and thermal stresses, resulting in lipid phase separation, cholesterol efflux, and protein migration in the plasma membrane [18]. These changes reduce motility, viability, and fertilizing capacity by disturbing membrane integrity [19]. The protective role of PRP may stem from NGF-mediated activation of MAP kinase signaling, which preserves membrane architecture [20], and IGF-1–mediated stabilization of acrosomal membrane proteins [22]. The protein content of PRP likely acts as a colloidal buffer, reducing osmotic stress and minimizing ice crystal formation during freezing and thawing [23].

However, excessive PRP 10% showed reduced efficacy. Higher concentrations may cause over-release of platelet-derived factors, increasing reactive oxygen

species (ROS) generation and oxidative stress. Elevated protein content can raise extender viscosity, interfere with cryoprotectant penetration, and create osmotic imbalance, leading to plasma and acrosomal membrane destabilization [5,7,8]. Overstimulation of growth factor pathways may also induce premature capacitation or acrosomal exocytosis, explaining the decline in post-thaw quality at higher PRP levels.

Differences in seminal parameters among breeds and individuals can result from factors such as age, nutritional status, season, hormonal balance, and overall reproductive health [24]. Despite these variations, the consistent superiority of 7% PRP supplementation across breeds in the present study suggests that moderate PRP inclusion effectively minimizes cryoinjury and enhances post-thaw semen quality.

CONCLUSIONS

The addition of PRP into the semen extender at different concentrations positively affected the freezability of semen in Barbari and Sirohi bucks. The addition of 7% PRP in the semen extender proved to be better in improving the post-thaw fertility of buck sperm, followed by 10%.

CONFLICTS OF INTEREST

The authors have declared that there are no conflicts of interest concerning this study.

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