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Comparing Fertility Outcomes of Washed and Unwashed Cryopreserved Semen in Beetal Goat Through Artificial Insemination

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The cryopreservation of buck semen facilitates the genetic improvement of goat breeds. The cryopreservation of buck semen needs attention to explore the efficient methods, as enzymes in buck semen may present harmful interactions with egg yolk-based extenders. The objectives of present study include comparison of semen quality parameters i.e., fresh vs frozen, unwashed vs washed (removal of seminal plasma) cryopreserved semen of Beetal bucks (Faisalabadi strain) and subsequent in-vivo fertilization (conception rate) using artificial insemination (AI). From government livestock farm (Jahanian, Punjab, Pakistan), three breeding bucks of same age group were selected and transported to semen production unit Qadirabad, Sahiwal Punjab, Pakistan. Each ejaculate of collected semen, after the gross and microscopic evaluation, was divided into two equal halves. One half was washed using a washing buffer before dilution and the other half was diluted directly. The French plastic straws (0.5 ml) were filled and sealed to cryopreserve the semen at -196°C. The cryopreserved semen was used to inseminate the estrus synchronized goats and conception rate was recorded using non-return rate to estrus and using serum progesterone level. The average semen volume ($P > 0.05$) and sperm count per ml was higher in first ejaculate while individual motility was higher in second ejaculate. The average post thaw motility for unwashed cryopreserved semen was higher (56.3 ± 1.92%) than washed semen (51.1 ± 1.57%). The estrus synchronization of Beetal does (n=60) was completed by two injections of prostaglandin (PGF-2α) at 10-day interval. Conception rate in inseminated goats using unwashed cryopreserved semen was lower (67.77%) as compared to washed frozen semen (68.88%) and fresh semen (73.3%). The fresh semen was collected and diluted at goat farm from the same three bucks that were used for semen collection for cryopreservation. It was observed that washed cryopreserved semen yields higher ($P > 0.05$) conception rate than unwashed semen which leads to understanding that washing improves the fertility rates, yet buck semen can also be cryopreserved without washing in common extenders and application of AI using cryopreserved semen in Beetal goats yields acceptable fertility rates. The study opens the aspect of comparing insemination techniques to further improve the effectiveness of this biotechnology.
Keywords: Goat, semen, cryopreservation, washed semen, conception rate

INTRODUCTION

Worldwide in goat production, Pakistan ranks 3rd largest country, with an estimated 87.0 million goats (Anonymous, 2024). The country hosts 36 goat breeds and has an annual population growth rate exceeding 3% (Khan *et al.*, 2008). This shows that goats are crucial components of countries agriculture economy, food security, and culture (Arief *et al.*, 2023; Derar *et al.*, 2023). Moreover, these animals have the potential to significantly contribute to the country's growth and development. Especially, Beetal breed, in Pakistan, is one of heavier breeds, known for its high potential in both milk and meat production (Khan *et al.*, 2008). However, to fully utilize this potential, we need to take actions to address the issues that are hindering their productivity and genetic improvement. One such issue is the limited access to quality breeding stock, especially the availability of superior bucks. This is where artificial insemination (AI) comes in as a practical assisted reproductive technology that can help optimize reproductive efficiency and accelerate genetic gain within and across herds. Reproductive efficiency of the animals determines the breeding stock efficiency and goat production sustainability (France *et al.*, 2024). The AI ensures the availability of good germplasm for the farmers (Qureshi

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et al., 2013) and enables them to select the best animals for breeding and thus promote genetic improvement of local goat breeds. Moreover, AI can maximize the number of offspring from a desirable sire and allow genetic exchange over wide geographical areas (Farooq *et al.,* 2023).

The success rate of AI heavily depends on semen quality and volume, both vary by different factors including breed of the bucks, their genetics, insemination methods (Gangwar *et al*., 2023) and physiological status (Shamsi *et al.* 2023). As male contributes genetic material to larger proportion of population (Yoon *et al*., 2022), male fertility is critical and semen quality evaluation is imperative for its role in genetic improvement. Other than male related factors, environmental conditions like photoperiod may also affect semen concentration, volume, percentage of abnormal spermatozoa, and progressive motility (Karagiannidis *et al*., 2000). Semen cryopreservation is more challenging in goats. Unlike cattle, diluting goat semen (neat or unwashed) with egg yolk (Purdy, 2006) and milk-based diluents can be detrimental to sperm cells (Kanmaz *et al.,* 2024). Constituents of seminal plasma, such as proteins secreted from bulbourethral gland, interact with milk components and inhibit sperm motility (Sias *et al*., 2005; Mara *et al*., 2007). The presence of yolk agglutinase and phospholipase in buck seminal plasma may hydrolyze yolk lecithin into lysophosphatides resulting in harmful effects on sperms by altering pH of diluent (Aziz *et al.,* 2023; Zou *et al*., 2022). The phospholipase A produces lysolecithin and fatty acids by interaction with egg yolk lecithin (Iritani and Nishikawa, 1963) which in turn reduce the semen quality and also cause acrosomal damage (Dorado *et al*., 2007). Using extenders with low levels of egg yolk significantly improves post-thaw semen quality compared to those with 20% egg yolk (Bispo *et al*., 2011). Additionally, washing buck semen to remove seminal plasma can enhance post thaw quality, particularly if extenders contain egg yolk and milk (Leboeuf *et al*., 2000). Another important aspect related to AI protocol in goats that can affect the performance of this technology is the site of semen deposition (Paulenz *et al*., 2005; Leethongdee *et al*., 2021) that can be intracervical, transcervical, intrauterine, and even vaginal insemination. The anatomical structure of the goat cervix differs slightly from that of bovines, requiring careful navigation of the cervix rings with an AI gun to deposit semen in the cervix or uterus (Salamon and Maxwell, 2000). Thawing of frozen semen is also a critical step in the AI process and affects fertility (Bezerra *et al*., 2012). Estrus synchronization is also a crucial component of successful AI programs. Without the use of ultrasonography, it remains difficult to assess the reproductive stage of the goats, particularly the follicle and corpus luteum (CL) stage and size. The CL in mammals is an endocrine gland that releases progesterone hormone during pregnancy (Jiemtaweeboon *et al*., 2011). Goats are usually considered short day breeders yet managemental changes and synchronization protocols help to modify breeding time. Various protocols are used for estrus synchronization worldwide. In Pakistani breeds, administering two doses of PGF2α at 10 or 12-day intervals has been reported to yield good synchronization results (Khanum *et al*., 2006).

In Pakistan, goat breeding relies on natural mating, with no commercial-scale alternatives currently in use. Some experimental work using fresh goat semen has been conducted (Mehmood *et al*., 2011; Qureshi *et al*., 2013). The studies on semen washing and cryopreservation of washed semen are lacking on Pakistani goat breeds, and this aspect has never been explored earlier in Pakistan. Which creates an immense need to document the possible interactions of seminal plasma enzymes and semen extender constituents and the resulting effects on semen quality and conception rates. Widespread application of this technology can change the outlook of goat farming completely. But for this purpose, efficient collection and cryopreservation of semen from quality bucks is required. AI protocols need to be standardized for the local breeds to ensure good results in the field conditions. Therefore, the objectives of this study are to document the semen characteristics of Beetal bucks, compare the washing and non-washing cryopreservation of semen and conception rate after AI using fresh and frozen (washed and unwashed) semen in estrus synchronized does.

MATERIALS AND METHODS

***Animals*:** The experiment was conducted on Beetal bucks (Faisalabadi strain). From government livestock farm in Jahanian, (30°2'30"N, 71°48'40"E) Punjab, Pakistan three breeding bucks (reproductively healthy) were selected and transported to semen production unit Qadirabad, Sahiwal (altitude: 564 ft above sea level; longitudes: 73^{o} & 74^{o} E; latitudes: 30^{o} & 31.15^{o} N), Punjab, Pakistan where the breeding bucks with good body condition score were trained for semen donation in artificial vagina and semen was collected, evaluated, processed and cryopreserved there. Reproductively healthy does (n=60) were estrus synchronized and inseminated at Livestock Experimental Station Allahdad, Jahanian, Punjab, Pakistan.

***Semen Collection*:** Semen collection was performed early in the morning on a three-day interval for fifteen days using sterilized artificial vagina (AV) specially designed for bucks. The number of ejaculates collected from each buck were 10, 7 and 6 from buck1, buck2 and buck 3, respectively. The AV was prepared to synch the natural conditions by pouring warm water (50-55°C) into it to keep inner temperature at 42°C. The tonicity and pressure inside AV were maintained at desirable level by blowing air inside of AV using air pump. The lubrication of AV using K-Y jelly, applied at inner liner of AV helped in providing required lubrication in artificial vagina for semen collection. The AV after preparation and including preheated graduated glass vial was covered properly by covert to avoid direct sunlight exposure.

***Evaluation of semen*:** The color and contamination were observed for each ejaculate of each buck and contamination free samples were processed for further evaluation. Volume of each ejaculate (1st and 2nd ejaculates of each buck) was recorded using graduated glass vials. Mass motility of fresh ejaculate was observed and recorded by placing an undiluted semen drop (5µl) using sterilized plastic straw on a pre-warmed glass slide without coverslip at 100X. The prewarmed (37℃) glass slides were placed on an electric stage warmed, phase contrast microscope, with controlled temperature, to prevent semen from cold shock, just after collection. The mass motility was graded at a score scale 1 to 5; as 1 representing the lowest and 5 represents the highest value for mass motility. The individual motility was observed by placing undiluted semen drop (5µl) on prewarmed glass slide and covered with a prewarmed coverslip at 200X using phase contrast microscope. The concentration of sperms in each ejaculate was measured using spectrophotometer.

***Washing of Buck Semen*:** For washing of semen Tris buffer was prepared. Each ejaculate was divided into two equal halves after gross examination and microscopic evaluation. The one aliquot was diluted in egg yolk-based extender and cryopreserved. The other aliquot was washed using a washing (Tris) buffer (Composition of Tris Buffer used for washing the semen was Tris (Hydroxymethyle) aminomethane 30.28 g, Citric Acid 17.5 g, Fructose 12.5 g, distil water 950 ml.) and after that it was diluted and processed for cryopreservation. For washing (removing seminal plasma), semen was diluted in Tris buffer at room temperature and centrifuged for 10 minutes at 3000 rpm, modified method of that presented by Islam *et al*., (2006). The supernatants were removed while sediment was diluted again in Tris buffer and centrifuged under same conditions. Sediments obtained after the second centrifugation were diluted using egg yolk-based extender. The composition of Tris Citric Acid Egg yolk extender (1 liter) includes Tris 24.20 g, Citric Acid 13.40 g, Fructose 10 g, distil water 730 ml, egg yolk 200 ml (20%), Glycerol 70 ml (7%), and Penicillin-streptomycin 1.0 g (the same extender composition was being used for bull semen dilution at semen production unit). The motility after dilution for both washed and unwashed semen was observed by placing undiluted semen drop (5µl) on prewarmed glass slide and covered with a prewarmed coverslip at 200X using phase contrast microscope.

***Semen processing and cryopreservation*:** The buck semen (both washed and unwashed) was diluted using an extender prepared with ingredients that include tris, citric acid, glycerol, egg yolk, distilled water and antibiotic (penicillin) (Ali *et al.,* 2024; Maulana *et al.,* 2024). The concentration of glycerol and egg yolk were 7% and 20% respectively. The semen was diluted based on the concentration of spermatozoa. The diluted semen was packed in 0.5ml French plastic straws at a final concentration of 80×10^6 spermatozoa per ml. The filled French straws (IMV®) were sealed with automatic filling and sealing machine. Semen filled sealed straws were placed in equilibrium condition at 4℃ for 4 hours. Later the filled sealed semen straws were plunged, for 7 minutes, on racks in vapors (four cm above surface) of liquid nitrogen to bring the straws at a temperature of -140℃. Then the semen straws were finally plunged in the liquid nitrogen for cryopreservation at -196℃ (Ramukhithi *et al*., 2011). The straws from both treatments were randomly selected for post thaw evaluation of cryopreserved semen. After 24 hours cryopreserved semen (one straw from each treatment) was thawed by dipping semen straw in warm water (37℃) for 30 seconds. The batch which has post thaw motility above 40% were finally placed in the goblet for cryopreservation in liquid nitrogen tank, until dispatched for use in artificial insemination process. However, the samples having below 40% post thaw motility were discarded.

***Estrus Synchronization*:** Beetal Faisalabadi goats (n=60) were estrus synchronized using Prostaglandin (PGF2α) analogue (cloprostenol sodium) intramuscular injections (0.5ml (dose adjusted according to weight) containing 125 µg/ml) repeated at 10-days interval (Khanum *et al*., 2006). The Prostaglandin (PGF2α) analogue was used with clear objective to bring goats at same reproductive stage as this hormone effectively regress the corpus luteum. The estrus stage in synchronized goats were detected in morning and evening through the apronized buck.

***Artificial Insemination in Goats*:** The vaginal speculum was prepared locally by smoothly cutting the test tube (Pyrex®) from blind end and attaching a light source. This glass tube speculum was prepared as an economical alternative to commercially available light-attached speculum to visualize external *os* in goats. Although stainless steel duck speculums were also available yet attaching light source in these were difficult and were not cost effective. The cervical external *os* was visualized using this vaginal speculum and, after having clear view of opened cervix intracervical insemination was performed. It was ensured to insert the smooth end of tube inside the vagina with proper lubrication to avoid any injury. The synchronized goats were randomly inseminated using fresh semen, washed and unwashed cryopreserved semen. The fresh semen was collected and diluted at goat farm from the same three bucks that were used for semen collection for cryopreservation.

***Conception rate*:** The inseminated goats were observed during days 20 to 22 post AI as non-return to estrus for estimation of conception rate. It was performed by exposing inseminated goats to the aproned breeding bucks and the status of these animals was confirmed by detecting serum progesterone level, as indicator of pregnancy, in goats that did not return to estrus after insemination. The blood sample of each inseminated goat was collected, serum was isolated, stored in icebox containers and were shifted to lab for storage at -20℃ till further processing in the lab. Progesterone level in serum was observed by enzyme linked immunosorbent

assay (ELISA) using enzyme immunoassay kit (as prescribed in manual of Biocheck®, USA).

***Statistical Analysis*:** The general linear model using the statistical package for social sciences (SPSS), computer software, was applied for analysis of data on semen quality parameters (both fresh and frozen). Binomial distribution (0= non-pregnant; 1= pregnant) was assumed for conception rate occurrence and, therefore, to test effects of three semen types (fresh, unwashed-frozen and washed-frozen) and bucks, logistic regression model was applied.

$$\mathbf{Ln}\,[\mathbf{P/1-P}] = \boldsymbol{\beta_0} + \boldsymbol{\beta_1}(\mathbf{Bucks}) + \boldsymbol{\beta_2}(\mathbf{Semen\ type}) + \boldsymbol{\varepsilon}$$

Where: **Ln** = Natural log, **P** = Probability of a doe getting pregnant, $[\mathbf{P/1-P}]$ = Odds ratio, odds of a doe getting pregnant, $\boldsymbol{\beta_0}$ = Intercept, $\boldsymbol{\beta_1}$ *and* $\boldsymbol{\beta_2}$ = Linear regression coefficients, $\boldsymbol{\varepsilon}$ = Random residual error.

Maximum likelihood principle was applied for parameter fitting and affects were tested, using Pearson method, by Chi-square.

RESULTS

***Volume*:** Difference in semen volume was significant ($P < 0.05$) between bucks while non-significant differences between ejaculate number ($P > 0.05$). However, volume has non-significant effects on washed semen motility both after dilution and also post thaw ($P > 0.05$). The variations in volume of the buck semen are presented in (Table 1 and Table 2).

***Sperm concentration*:** The sperm concentration per ml of semen varied among the bucks and ejaculates with minimum concentration 812 million and maximum 2176 million sperms per ml (Table 1 and Table 2). The spermatozoa concentration and post dilution motility differ significantly ($P < 0.05$) due to changes in ejaculate volume. In the present study, a highly significant ($P < 0.05$) effect of buck was observed on sperm concentration while non-significant ($P > 0.05$) effect of ejaculate number on concentration was observed.

***Mass Motility*:** The mass motility recorded in bucks varied among bucks and between ejaculates of same buck (Table 1 and Table 2) presenting the minimum 2 and maximum 3.5 scores for mass motility. The mass motility did not differ significantly ($P > 0.05$) between bucks and ejaculates.

***Individual Motility*:** The Individual Motility (IM) in buck semen was 65% (minimum value) and 85% (maximum motility). The differences in IM between bucks and between ejaculates was ($P > 0.05$). Yet, motility after dilution was significantly ($P < 0.05$) affected by IM. While non-significant ($P > 0.05$) effect of IM on post thaw motility in washed semen was observed (Table 3). Semen quality primarily can be assessed through individual motility of spermatozoa including progressive motility and relevant parameters.

***Motility after dilution*:** Motility of semen samples after dilution was examined and it differed ($P > 0.05$) between bucks and between ejaculate number both in washed and unwashed diluted semen.

***Post thaw motility*:** The motility of spermatozoa differs due to the thawing procedure when compared to fresh and diluted semen motility. There was variation in post thaw motility between bucks and between washed and unwashed semen (Table 1 and Table 2). For unwashed frozen semen, post thaw

Table 1. Values of seminal parameters in of all bucks (Mean ± S.E)

Parameters		Buck 1	Buck 2	Buck 3
Volume per ejaculate (ml)		2.17±0.18[a]	1.53±0.38[b]	1.08±0.24[c]
Sperm Concentration*		1118.90±76.9[a]	1955.70±261[b]	1788.70±90[c]
Mass Motility (0-5 score)		2.65±0.10[a]	2.85±0.23[a]	3.00±0.13[a]
Individual Motility (%)		80.00±0.00[a]	77.80±2.14[a]	80.80±2.39[a]
Motility after dilution	Unwashed	74.00±1.63[a]	71.40±3.03[b]	71.70±2.79[c]
(%)	Washed	68.00±3.09[a]	69.30±3.35[b]	69.20±3.52[c]
Post Thaw Motility (%)	Unwashed	63.00±2.49[a]	49.30±1.70[b]	53.30±3.33[c]
	Washed	52.00±2.49[a]	50.00±2.18[a]	50.80±3.96[a]

*(millions per ml)

Table 2. Seminal parameters of bucks for 1st and 2nd ejaculates (Mean ± S.E)

Parameter	1st Ejaculate	2nd Ejaculate	Average
Volume (ml)	1.8 ± 0.26[a]	1.5 ± 0.18[a]	1.6 ± 0.22
Sperm concentration*	1590 ± 172[a]	1482 ± 147[a]	1548 ± 117.3
Mass motility (score 1-5)	2.75 ± 0.12[a]	2.88 ± 0.14[a]	2.8 ± 0.10
Individual motility (%)	78.6 ± 1.33[a]	81.1 ± 0.74[a]	79.6 ± 0.88
Unwashed diluted motility (%)	72.1 ± 2.01[a]	73.3 ± 1.44[b]	72.6 ± 1.33
Washed diluted motility (%)	68.6 ± 2.48[a]	68.9 ± 2.86[b]	68.7 ± 1.84
Post Thaw motility (%)	Unwashed semen	56.3 ± 1.92	
	Washed semen	51.1 ± 1.57	

*(millions per ml)

Table 3. Correlation between semen quality parameters

Parameters	Conc.	IM	MM	MD	MWD	PTD	PTW
Vol.	-0.36*	0.32	0.03	0.46*	0.19	0.42*	0.17
PTW	0.17	0.15	-0.20	0.29	0.60**	0.34	-
PTD	-0.28	0.24	-0.01	0.64**	0.42*	-	-
MWD	0.35	0.40*	0.22	0.51*	-	-	-
MD	0.19	0.67***	0.43*	-	-	-	-
MM	0.14	0.49*	-	-	-	-	-
IM	0.19	-	-	-	-	-	-

* = (P < 0.05), ** = (P < 0.005), *** = (P < 0.0005); Vol=Volume; PTW=Post thaw Washed; PTD=Post thaw Diluted; MWD=Motility Washed Diluted; MD=Motility Diluted; MM=Mass motility; IM=Individual Motility; Conc=Concentration

motility was significantly (P < 0.05) different between bucks while for washed semen there was non-significant (P > 0.05) difference between bucks.

***Estrus synchronization*:** The goats included in estrus synchronization protocol (n=60) were observed for estrus signs and 48 goats expressed signs of estrus (as detected by the aproned buck) yielding 80% synchronization.

***Conception rate*:** The conception rate evaluated through non return to estrus and further confirmed by serum progesterone level were presented in Table 4 and Table 5. There was a non-significant difference (P > 0.05) of conception rate between semen collected from different bucks. The difference of conception rate between fresh diluted and cryopreserved semen inseminated was non-significant (P > 0.05).

Table 4. Conception rate (%) using fresh, washed and unwashed cryopreserved semen

Treatment	Buck #	Conception rate	Average
Fresh Semen	Buck 1	60.0[a]	73.30[a]
	Buck 2	100.0[a]	
	Buck 3	60.0[a]	
Washed Frozen	Buck 1	66.7[a]	68.88[a]
Semen	Buck 2	60.0[a]	
	Buck 3	80.0[a]	
Unwashed Frozen	Buck 1	60.0[a]	67.77[a]
Semen	Buck 2	83.3[a]	
	Buck 3	60.0[a]	
Overall average	All bucks	70.0	

The natural differences of buck and bull semen biochemical attributes suggest that extenders containing egg yolk or milk may have different effects on buck semen and resultant conception rates. Yet, in present study, washing of semen did not generate significant differences of conception rates as compared to neat (unwashed) cryopreserved semen.

The odds ratio (odd of being pregnant) is presented in following terms.

$$\text{odds} = \text{p(buck, semen type)}/1 - \text{p(bucks, semen type)}$$

The odd ratio for bucks ($e^{b_1} = e^{0.01385} = 1.01$) represents proportional change in chance of being pregnant (odds) due to change of bucks while considering semen type as constant. The current odd ratio of bucks presents 1.00% change/increase in probability of occurrence of pregnancy in inseminated does with changing bucks while taking semen type as constant.

The odd ratio ($e^{b_2} = e^{0.12071} = 1.13$) for semen type is proportional change in chance of being pregnant (odds) due to change of semen type when considering bucks as constant. The current odd ratio of semen type presents 13.00% increase in probability of pregnancy occurrence in inseminated does while bucks were taken as constant.

DISCUSSION

***Volume*:** In the present study, the semen volume for each buck varies and also for each ejaculate, these variations may be attributed to the genetic factors, testicular attributes, libido and individual buck adaptability to semen collection process as all these bucks were being used for natural mating as breeding bucks at the farm. These variations have been documented in different studies. Average semen volume was 0.5 ± 0.02 ml in adult bucks (Bitto and Egbunike, 2012) while 2.7 ml average volume of semen per ejaculate (Rajuana *et al*., 2008). The average semen volume 1.2 ± 0.02 ml was of three different breeds (Karagiannidis *et al*., 2000). The average semen volume per ejaculate in three goat breeds including Local black, Cyprus and crossbreed (Cyprus X local black)

Table 5. The ANOVA values for this experiment

Variables	SOV	Coef.	S.E Coef.	Z	P value	Odds Ratio	95% CI	
							Lower	Upper
Conception rate	Const.	-10.21400	12.67810	-0.81	0.420			
	Bucks	0.01385	0.16162	0.86	0.391	1.01	0.98	1.05
	Semen	0.12071	0.39757	0.30	0.761	1.13	0.52	2.46

SOV=Source of Variation; Coef. = Coefficient; CI = Confidence interval

were 1.72±0.08, 1.65±0.05, 1.69±0.09 ml respectively (Ali *et al*., 2024). Apu *et al*., (2008) reported significant ($P < 0.05$) difference in ejaculate volume. The volume of semen per ejaculate between bucks differ significantly. The average semen volume in bucks was 0.43 ± 0.01 ml (Kardani *et al*., 2024) while in another study, average semen volume in winter 1.14 ± 0.1 and summer was 1 ± 0.1 ml (Mohamed *et al*., 2023) in mature bucks. The variation from present study may be due to breed effects, age or managemental variations.

***Sperm concentration*:** The sperm concentration in ejaculated semen of bucks is affected by factors including testicle size, testicle shape, testicle lobular condition, testicle circumference, libido, semen donation frequency, ejaculate number, and also due to conditions during collection procedure including AV temperature and lubrication and collection time. These factors alone or in combination have been documented by different researchers. The significant variation in total sperm count among bucks was observed by Kardani *et al*., (2024). Highly significant ($P < 0.01$) difference among the bucks semen concentration was observed (Apu *et al*., 2008). Likewise, Silvestre *et al*., (2004) reported sperm concentrations of 2.2, 2.8, and 2.2 (billion per ml) while 3.6 ± 0.04 (billion per ml) of ejaculate were reported by Karagiannidis *et al*., (2000). The semen sperm concentration 3796.88 ± 34.88 million/ml and total sperm count 1624.61 ± 47.46 million were recorded in Surti bucks (Kardani *et al*., 2024). The variation in sperm concentration in winter season 1599.6 ± 1.5 and summer season 1139.8 ± 2.4 were evident (Mohamed *et al*., 2023).

***Mass Motility*:** The mass motility in buck semen varies due to factors including buck related factors (genetics, body condition score, libido), collection procedure (gentleness of applying artificial vagina, lubrication, maintenance of temperature, false mounting, ejaculate number), collection time and frequency. These values of mass motility observed in present study coincide with values reported in literature. Average mass motility of one buck was 3.8 ± 0.2 while for the other buck it was 3.7 ± 0.2 (Mishra *et al*., 2010). Average mass motility of 3.6 ± 0.08 for buck semen was reported by Bitto and Egbunike, (2012) and 3.66 ± 0.06 by Kardani *et al*., (2024).

***Individual Motility*:** The variations in seminal attributed may be due to individual genetic variations of bucks and reproductive capacity of each buck as managemental conditions were similar for all three bucks during the semen collection and handling process. Keeping in view different factors variations in individual motility may exist. In different studies, the variations in IM of goat semen were reported. The difference of IM was significant ($P < 0.05$) between bucks (Apu *et al*., 2008). These differences may be due to the breed, age or other managemental aspects. Likewise, there were significant differences ($P < 0.05$) between ejaculates within the same male in terms of sperm quality (Dorado *et al*., 2010). Similar range of IM (74.6 ± 0.85%) of semen ejaculate from healthy buck was reported by Bitto and Egbunike, (2012). While average IM of fresh semen from bucks was 87.5 ± 4.62% (Kozdrowski *et al*., 2007).

***Motility after dilution*:** The dilution may affect the individual motility (IM) of the sperms, either due to the constituents present in the extenders or because of semen handling techniques before and after dilution and washing process. Previous studies by Apu *et al*., (2008) presented significant difference ($P < 0.05$) between bucks for IM in diluted semen. Islam *et al*., (2007) reported post dilution IM of semen in range of 70.00 ± 2.89 to 73.33 ±1.67%.

***Post thaw motility*:** Post-thaw motility varies because of multiple factors ranging from animal to lab process, yet in present study these variations may be due to genetic differences of bucks as an individual effect and due to washing and non-washing processes before semen cryopreservation. Different studies reported the post thaw motility of buck semen in different ranges, these differences may be attributed to the factors listed above or even managemental conditions and environmental factors directly affecting animal's health and comfort. The seasonal effects on post thaw quality of semen have been reported by Kulaksiz *et al*., (2020). In washed semen, non-progressive sperm cell motility was 33.9% while progressive motility was 49.3% (Ramukhithi *et al*., 2011). Post thaw motility reported by Kulaksiz *et al*., (2013) in three goat breeds, including Angora (41.6 ± 1.05%), Kilis (71.6 ± 1.05%) and Saanen (61.6 ± 1.05%) with an average (58.2%) for all three breeds. The range of post thaw motility of cryopreserved buck semen was between 55% and 65% (Gacitua and Arav, 2005).

***Estrus synchronization*:** The success rate of estrus synchronization in goats particularly depends on the factors including physiological stage of animal, reproductive physiology, ovarian status for follicle and CL, animal's health, breed differences, feeding and management of animals even though same protocols of synchronization have been implemented. The anatomical difference between size of large and small ruminants creates limitation in sheep and goats to palpate ovarian structure and so left with the option of ultrasonography for exact calculation and precise ovarian stage identification yet the breeding records and proper management of animals help to identify the animals which are not pregnant but in an anestrous stage. Keeping in view the estrus cycle of animal, targeting CL and follicle stage for estrus synchronization is important and it yields different results in different experiments based on efficiency of protocol and also selection of animals for estrus synchronization. In different experiments 95% estrus synchronization using prostaglandin F2α was observed (Amarantidis *et al*., 2004; Khanum *et al*., 2006) while 88.14% expressed signs of estrus (Dea *et al*., 2023). The findings of current study were in line with similar to reported by Omontese *et al*., (2013). The difference of results of present and previous studies may be attributed to factors such as

breed, age, parity, physiological stage, reproductive history of animals, health status and managemental conditions including housing, watering, feeding and space availability for each animal allowing it to express normal behaviors of each physiological stage.

***Conception rate*:** Although, conception rates may differ due to multiple factors both of male and female origin and also due to factors involved in semen handling, semen quality, semen state (fresh, frozen, washed, unwashed), AI techniques, site of semen deposition and post AI management of female animals and even the environmental conditions including any stressor like temperature humidity index or compromised welfare conditions. Different previous studies have reported different conception rates using artificial insemination. In estrus synchronized goats inseminated, the conception rate was 53.9% (Khalifa and El-Saidy, 2006). The fertility rate among goats inseminated with washed semen was 70.3% and for unwashed semen 73.5% (Roca *et al*., 1997). In Black Bengal goat, fresh dilutes semen resulted in an average kidding rate of 58.9% (Apu *et al*., 2008) while in another study conception rate was 59% after AI using fresh diluted (Pandey *et al*., 1985). Average conception rates, after inseminating goats with fresh diluted semen of different bucks, were 50%, 58%, 67% while bucks in AI in goats while frozen thawed semen resulted in 37%, 33%, 53% conception rates, however, conception rate for pooled cryopreserved semen was 42% (Gacitua and Arav, 2005). Average conception rate in goats inseminated using frozen thawed semen was 54% (Gibbons and Cueto, 2011) and 48.7% pregnancy rate after AI in goats (Arrebola *et al*., 2012). The conception rate variations as a result of artificial insemination can also be due to different time of insemination using fresh semen (Murtaza *et al*., 2020) along with other factors related to semen, estrus and technique.

The vaginal insemination in Norwegian dairy goats using liquid semen (200 x 10^6 sperm) resulted in 25th day no-return rate of 85.5% and a kidding rate of 74.3% (Paulenz *et al*., 2005). However, at same sperm concentration of frozen thawed semen, after insemination the non-return rate was 37.3% on 25th day while kidding rate was 24% (Nordstoga *et al*., 2011). The variation from present study may be due to the site of deposition of semen and type of semen inseminated. The impact of the breed may also be the reason for variation in fertility and kidding rate. Single insemination resulted in non-significant differences (Nordstoga *et al*., 2010) of fertility rate as using frozen thawed semen (400 x 10^6 sperms/straw) and double insemination of frozen thawed semen (200 x 10^6 sperms/straw). The pregnancy rate in Murciano-Granadina goats after post-cervical insemination using frozen thawed semen was 57% (Salvador *et al*., 2005). However, kidding rate after trans-cervical insemination in goats followed by frozen-thawed semen was 71% and 1.76 litter size (Sohnrey and Holtz, 2005). These reported results are in accordance with present study, presenting acceptable results of artificial insemination in goats using frozen thawed semen. The higher fertility rates in present study may also be due to higher concentration of spermatozoa. However, the variations may be attributed to factors such as but not limited to, age of females, parity, semen handling, techniques of insemination, site of semen deposition, insemination time and hygienic measures opted during AI.

The current study opens the opportunities to further explore the biochemical properties of buck semen, particularly of Pakistani goat breeds, additionally to study the effects of varying composition of semen extenders, breed effects, semen deposition sites and fixed time artificial insemination in goats for precision in goat breeding and applications in genetic improvement through dissemination of superior buck genetics. The AI implementation in goats at large scale will create huge economic impact in terms of creating technical human resources, availability of good genetics, and growth of allied business.

Limitations of study implementation: The present study only encompasses a few aspects of semen cryopreservation and artificial insemination. Although with great need for AI in field, the lack of awareness of AI availability, lack of trained human resources and lack of cryopreserved semen are the factors creating hindrance in general application and widespread adoption of this biotechnology in Pakistan at present. These areas may be improved on a priority basis for the benefit of the goat breeders in specific and livestock industry in general.

***Conclusion*:** The buck semen can be cryopreserved successfully using an extender containing 20% egg yolk, even without washing. Artificial insemination resulted in acceptable conception rates using fresh and frozen (washed and unwashed) semen with non-significant differences in Beetal goats in Punjab, Pakistan. The cryopreservation of buck semen will help in efficient breeding program implementation and in conservation programs and the technique of AI will help in disseminating the best male genetics at a rapid rate for genetic improvement of goat breeds.

***Conflict of Interest*:** The Authors declare that there is no conflict of interest.

***Authors' contribution Statements*:** SI and MSK conceived the idea and designed the study. MSK, AUH and SAB supervised the research. SI conducted research. FR, MSR and FUH helped in execution of research work. SI and MSK analyzed the data and prepared manuscript. All authors critically edited the manuscript.

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