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Research Article

The Influence of Glucose and Fructose as Energy Source in Semen Extender on the Quality of Chilled Canine Semen Stored at 4 °C

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ABSTRACT

The experiment was designed to assess the effects of alternative energy sources on chilled semen, extended for use over a longer period of time. Fructose and glucose were used as energy sources to evaluate the sperm viability when semen is chilled for a longer period of time. Five dogs were selected, and semen was collected at five-day intervals. Extender was added only to the sperm-rich fraction and evaluated for seminal parameters and sperm morphology at 0, 24, 48, and 96 hours at 4°C. The results showed that the addition of glucose to the semen extender resulted in sperm motility of 70.6% ± 2.54% and acrosomal integrity of 47.6% ± 3.11%. In comparison, fructose resulted in 74.2% ± 2.50% motility and 52% ± 3.10% acrosomal integrity after 24 hours of cold storage. These findings indicate significantly improved sperm motility and acrosomal integrity with the fructose-based semen extender compared to glucose. In conclusion, the addition of fructose to the semen extender has a positive influence on sperm quality.

Keywords: Motility, Acrosomal Integrity, Fructose-Based Semen Extender, Glycerol-Based Semen Extender.

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INTRODUCTION

Sperm reduces its metabolic activity when cooled and preserved, primarily through controlled motility. Components of the extender are source of nutrition for the sperm to maintain the stability and prolong sperm survival (Wysokińska, 2025). Canine semen with good motility can be stored for up to 8 days if the temperature is maintained between 4℃ and 5℃ and the fertile period may be preserved for up to 10 days (Verstegen et al., 2005). However, cooling semen to 1℃ can cause cellular injury due to cold shock, either by altering cellular functions or disturbing cellular structures. Glucose and fructose significantly affect metabolic activity in different ways (Martínez & Salinas, 2021). Their main effect in canines is to influence sperm progressive motility and movement patterns (Martínez & Rivera, 2022). Cold shock reduces the proportion of motile spermatozoa and can lead to cellular death (Amann & Pickett, 1987). Compared to other species, canine semen is more tolerant of cold shock (Johnston et al., 2007). In terms of sensitivity to cold shock, the sperm of dogs, cats, horses, and humans can tolerate it relatively well, whereas the sperm of goats, cattle, and sheep show moderate tolerance. Boar sperm, however, is extremely sensitive to cold shock (Watson, 1986). Cooling increases sperm membrane permeability, which results from disturbances in specific spermatozoal protein channels. Chilling leads to reduced cell functionality and ultimately cell death, largely due to the regulation of calcium uptake channels.

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The influx of calcium during cooling causes alterations in capacitation, which can trigger fusion events between the acrosomal membrane and the sperm plasma membrane (Barbas & Mascarenhas, 2009). When temperatures drop below freezing, ice forms outside the cell through water crystallization, leaving solutes more concentrated altering the pH.

Various semen extenders have been utilized over the past several decades to preserve sperm quality during storage. Among these, Tris-citrate egg yolk (EY) extenders supplemented with either glucose or fructose have consistently demonstrated superior results in terms of sperm viability and motility under laboratory conditions (Linde-Forsberg, 1995; Rota et al., 1995; Iguer-Ouada and Verstegen, 2001; Peña et al., 2006). Due to their cost-effectiveness and ease of use, the preservation of semen in a chilled liquid state at 5 °C is widely recommended by artificial insemination (AI) practitioners. Compared to frozen semen, chilled semen generally yields improved AI outcomes (Linde, 2001). Additionally, the inclusion of egg yolk in canine semen extenders has been shown to enhance all parameters of sperm motility during storage at 4 °C (Verstegen et al., 2005). Experimental findings have demonstrated that high-quality spermatozoa can be preserved for up to 10 days when an appropriate energy source is maintained within the extender medium. Goericke et al. (2012) supported the earlier findings of Iguer-Ouada (1999), validating the viability of extended chilled canine semen for up to 10 days, with only minor variations in the results. In a study, utilizing a Tris-glucose egg yolk (EY) extender, a low concentration of live and motile spermatozoa was observed by day 16 post-collection, indicating a gradual decline in sperm quality. Nevertheless, this outcome underscores the efficacy of the extender and the beneficial role of egg yolk in preserving sperm viability at 4 °C. The findings further confirmed that the EY-based extender is among the most effective media for maintaining sperm motility under chilled conditions (Farstad, 2009). Egg yolk, although not a chemically defined compound, serves as a biological source of essential components such as vitamins, glucose, antioxidants, phospholipids, and proteins, all of which contribute to the maintenance of cell membrane integrity. Additionally, Nguyen et al. (2019) reported enhanced results when using fructose-supplemented extenders for chilled canine semen, suggesting the energy source plays a significant role in extender performance.

The present study was designed to evaluate the influence of glucose and fructose as energy sources in semen extenders on the quality of chilled canine semen stored at 4 °C. Specifically, the objectives included assessing sperm motility and acrosomal integrity in semen extenders based on glucose and fructose.

MATERIALS AND METHODS

Extender preparation and semen chilling

Extenders used for semen freezing or cold storage must contain nutrients as an energy source, cryo-protectant to protect from cold shock while decreasing the temperature, buffer against harmful changes in pH, maintain osmotic pressure / concentration of electrolytes and prevent bacterial growth. This standard composition contains EY, glycerol, buffer, sugar source and antibiotics. A typical extender formulation includes egg yolk (EY), glycerol, buffering agents, a sugar source, and antibiotics. In the present study, a laboratory-formulated extender was developed to preserve canine semen under cold storage conditions. The extender was prepared using a combination of glucose and fructose as energy sources, with Tris and citric acid as buffering agents, as shown in Table 1. Unfertilized hen eggs were utilized as the source of egg yolk. All chemicals were handled under dry, non-humid conditions to preserve their stability and efficacy. The preparation process was conducted under a laminar flow cabinet, which provided a sterile working environment by maintaining a constant stream of filtered air to prevent contamination. Laboratory glassware and plasticware were dried using a drying oven to eliminate residual moisture without compromising material integrity. Following extension, the semen was chilled via refrigeration and maintained at 5 °C for 96 hours. Semen samples were then evaluated for morphological parameters. In the control group, no sugar was added to the extender. In contrast, experimental group A used an extender containing glucose, and group B used an extender containing fructose, with both sugars provided in equal concentrations. A rapid cooling rate of 1 °C per minute was employed, in accordance with the protocol recommended by Bouchard et al. (1990).

Acrosomal integrity

Sperm that are unable to undergo the acrosome reaction properly are incapable of fertilizing the ovum. Acrosomal integrity is essential for fertilization, making it an important parameter in semen evaluation for assessing the breeding fitness of a stud dog. In this study, acrosomal integrity in extended chilled semen was evaluated using a staining technique. A dual-staining method was employed, utilizing Trypan Blue, Formal-Neutral Red (FNR), and Giemsa stain. The FNR solution was prepared by combining 86 mL of 1N HCl, 14 mL of 37% formalin, 0.2 g of

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Neutral Red, and distilled water to a final volume of 100 ml. The FNR solution was stored at 4 °C. A 5 µL aliquot of extended semen was mixed with an equal volume of Trypan Blue and allowed to air dry on a microscope slide. FNR was then applied at room temperature for 2–3 minutes, followed by washing with distilled water. The same specimen was subsequently treated with Giemsa stain for 2.5 hours and washed again before analysis.DPX mountant, a mixture of distyrene, a plasticizer, and xylene, was applied to inhibit stain fading due to its antioxidant properties. The prepared slide was then examined under a microscope. Sperm with white-colored heads were considered to have intact acrosomes, indicating acrosomal integrity, while blue-stained sperm were classified as non-viable due to damage to the acrosomal membrane (Figure 1). Trypan Blue distinguishes live from dead cells by selectively staining only those with compromised membranes. Because it cannot penetrate the intact membranes of viable cells, it serves as an indicator of cell viability. The staining mechanism of Trypan Blue is based on its negatively charged nature, which restricts its entry into living cells.

Table 1. Composition of Tris-Citrate Glucose (A) and Tris-Citrate Fructose (B) Extenders for Canine Semen Cooling.

Component	Extender A (Glucose)	Extender B (Fructose)
Tris (g)	3.025	3.025
Citric Acid (g)	1.700	1.700
Penicillin (g)	0.100	0.100
Streptomycin (g)	0.100	0.100
Glucose (g)	0.180	–
Fructose (g)	–	0.180
Egg Yolk (mL)	20.00	20.00

Note: Both extenders were prepared using identical base components, with the only variable being the sugar source—glucose in Extender A and fructose in Extender B. Egg yolk served as the cryoprotectant and nutrient source.

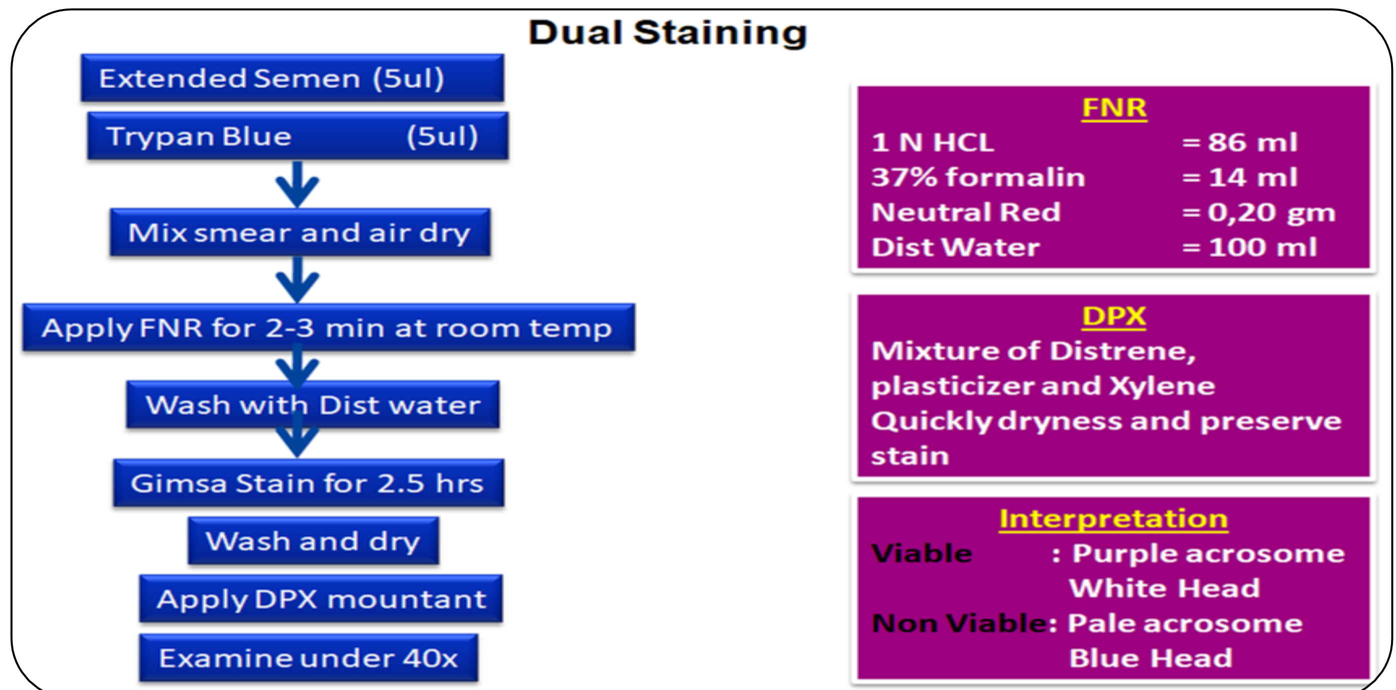

Figure 1. Staining technique used for acrosomal integrity in extended chilled semen.

Inclusive criteria

The semen of dogs was collected from the dogs at NARC, Islamabad Pakistan.

Exclusive criteria

The semen of dogs from other sites of the Islamabad was not considered in the present study.

Ethical approval

The study approval received from the Research Ethical Committee of NARC, Islamabad Pakistan.

Statistical analysis

The data were subjected to statistical analysis using mean values with standard deviations. Analysis of variance (ANOVA) was performed to evaluate differences among treatments (n = 5), using SPSS version 21 statistical software. Following the ANOVA test, Pearson correlation analysis was conducted to determine the associations between the treatments.

RESULTS

Semen was collected from five Pointer dogs to compare the effects of two extenders on chilled semen. The composition of the two extenders was identical, except for the energy source: Tris-citrate-glucose (TCG) and Tris-citrate-fructose (TCF). The energy source was varied to evaluate its effect on sperm motility and acrosomal integrity at 0, 24, 48, and 96 hours post-collection during storage at 5°C, as reported in Table 2. Statistical significance was determined using a two-way ANOVA test at a significance level of α = 0.05 to compare the two parameters in dogs. For accuracy, the data were expressed as mean ± standard deviation (SD). A p-value less than 0.05 (P < 0.05) was considered statistically significant, indicating a meaningful relationship between the variables. Conversely, a p-value greater than 0.05 indicated no significant relationship, as shown in Table 2.

Table 2. Effects of energy source on motility and acrosomal integrity of the semen.

Time	Motility %		Acrosomal Integrity %		2 Way ANOVA Test	Remarks
(hr)	TCG	TCF	TCG	TCF	P < 0.05	Significant/ non-significant
0	81.6 ± 0.28	82 ± 0.20	95 ± 0.28	94.6 ± 030	0.0001	Significant
24	70.6 ± 2.54	74.2 ± 2.50	47.6 ± 3.10	52 ± 3.11	0.0001	Significant
48	59 ± 1.83	61.6 ± 1.80	42.4 ± 2.26	45.6 ± 2.20	0.0001	Significant
96	38.2 ± 3.39	43 ± 3.30	32 ± 2.68	35.8 ± 2.60	0.0001	Significant

Two Way ANOVA test (α significance at 0.05) ± S.D.

The comparison between energy sources to evaluate the effect on motility and acrosomal integrity at 0, 24, 48 and 96 hrs of collection when stored at 5℃ as shown in figure 2 and figure 3.

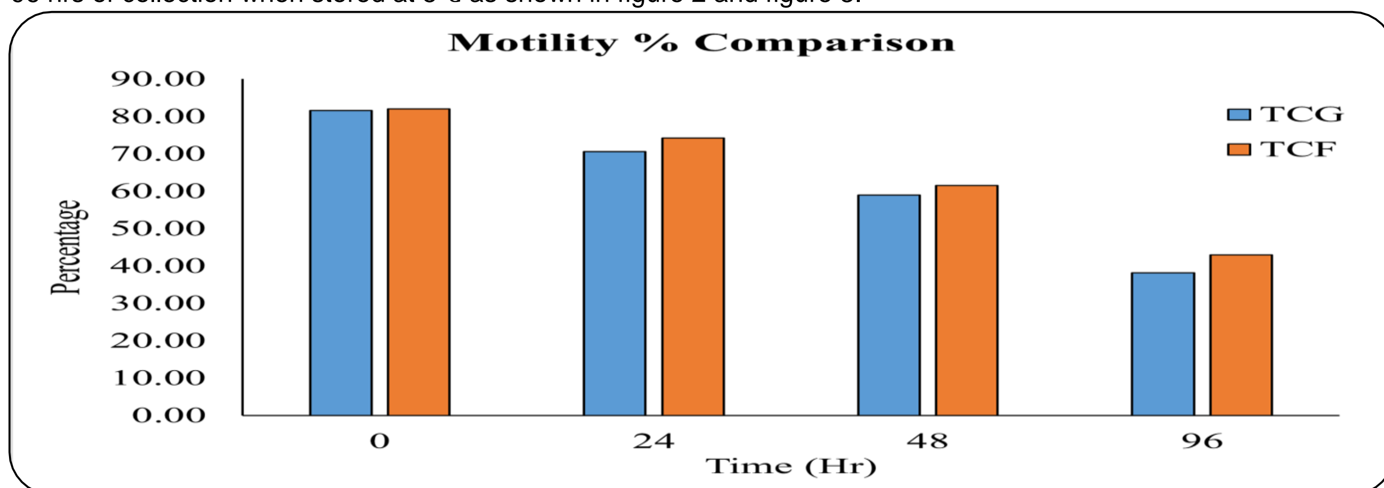

Figure 2. Effect of varying semen extenders on sperm motility %.

The correlation between the two parameters (Sperm mobility and Acrosomal integrity) determined in glucose-based semen extruder and fructose-based extruder of dogs were positive correlation with each other as represented in table 3.

After Two-way ANOVA test for further analysis, Pearson correlation between different parameters of semen extruders of dogs was performed to determine the association within the samples and it indicated that Sperm mobility and Acrosomal integrity in glucose semen extruder and fructose semen extruder have strong relationship between them as reported in table 4.

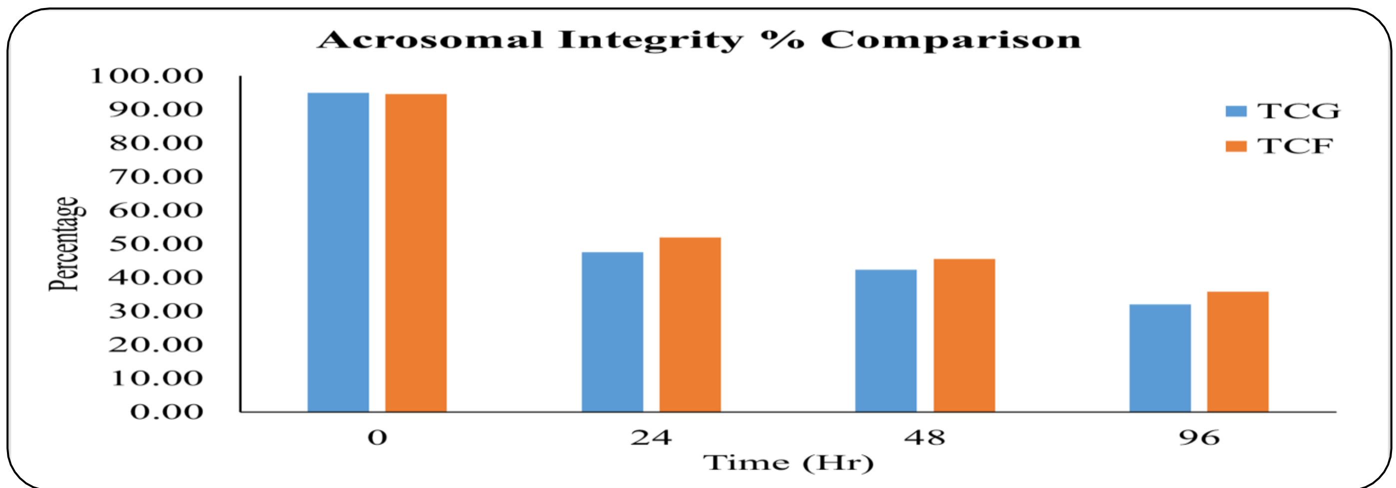

Figure 3. Effect of varying semen extenders on acrosomal integrity %.

Table 3. Correlation between different parameters in Glucose based semen extruder and fructose-based semen extruder.

Pearson Correlation (P < 0.05)

Parameters	Sperm Mobility		Acrosomal Integrity	
	TCG	TCF	TCG	TCF
TCG	1			
TCF	0.840327	1		
TCG	0.998235	0.814355	1	
TCF	0.853732	0.999533	0.829368	1

Table 4. Correlation between different parameters in Glucose and fructose-based semen extruder.

Parameters		Correlation	Two Way ANOVA Test	Status (P<0.05)
Sperm Mobility	TCG vs TCF	0.84	0.000	Significant
Sperm Acrosomal	TCG vs TCF	0.82	0.000	Significant

Two Way ANOVA test (α significance at 0.05)

DISCUSSION

Egg yolk was used as nutritional support that contains phospholipids and low-density lipoproteins (LDL), provides protection to the sperm membrane. Most diluents for canine semen contain 10-20% egg yolk. Various studies confirmed successful cryopreservation and chilling with LDL extracted from egg yolk in canine and bull semen (Amirat et al., 2010). In another experiment, Barbas and Mascarenhas, (2009) determined the protective components in egg yolk are phospholipids (lecithin) and LDL adhere to the sperm membrane and form an interfacial layer. Watson (1976) demonstrated variability of egg yolk composition on the basis of nutrients being supplied to the chicken. Eggs used in current experiment were obtained from a layer farm where the birds were being maintained on commercially prepared feed. All eggs were used from the same source to avoid variation in egg yolk composition. It revealed that egg yolk contains progesterone. Presence of progesterone in egg yolk interfere capacitation of spermatozoa. Aitken and McLaughlin (2007) examined the effects in cattle, horses and dogs stating that capacitation of sperm may be a cause of acrosomal destabilization (Witte & Schäfer-Somi, 2007). Another commonly used protein source for semen extenders is whole or skimmed milk (Rota et al., 2001).

Each ejaculate was extended with TCG and TCF extenders. It is important to maintain the extender as isotonic with the sperm and should have a pH of 6.9 to 7.1. Both the extenders were protected from bacteria by adding antibiotics. Ejaculates even from healthy animals are sometime contaminated with bacteria. However, there is a considerable concern about the antibiotic-resistant strains of bacteria (Catry et al., 2010). The semen was diluted at 37℃ in extenders in the ratio 1:1 and cooled with fast cooling to 5℃ temperature by fast cooling method. Fast cooling rate was maintained by dropping 1℃ per minute till it reached to 5℃. Results revealed that fructose is better energy source if compared with glycerol in case of most important in vitro fertility parameters i.e. motility and

acrosomal integrity. Both the parameters declined at faster rate between 24 – 48 hrs intervals.
Acrosomal integrity in chilled semen was evaluated in two different extenders and found the fructose as a best energy source if semen is stored at 4 – 5℃ for up to 96 hrs. Motility was also better in TCF as compared to TCG extender. Capacitation causes a change in the pattern of spermatozoa movement which becomes hyperactivated. Capacitation and hyperactivation of sperm cells are prerequisites for the acrosome reaction to occur, which is essential for penetration of the *Zona pellucida* and ultimately the fertilization (Ho and Suarez 2001b; Suarez 2008).
Treulen et al. (2012) used EYT extender with addition of SP (seminal fluid) and PF (prostatic fluid) in two different groups and indicated significant decrease in motility after 48 hrs when the ejaculate was stored at 5℃ over 72 hrs. Current study did not show sudden decrease in motility due to addition of glucose and fructose as energy source and separation of third fraction to avoid mixing of PF. There is gradual decrease in motility from 0 – 96 hrs in chilled semen (Table - 2). However, fructose found even better energy source as motility remained better in all ejaculates extended with TCF extender. Fructose also proved better in case of acrosome integrity percentage. Better results of TCF could have clinical applications for the cooling and shipping of canine semen. It indicated the reduction in motility preserves the functionality of semen by reducing energy consumption for a prolonged period of time. This Positive or negative effects PF to SRF ratio is yet to be explored on cooled shipped canine semen. However, present study has revealed that extenders with energy source has better effects on chilled sperm once compared with previous studies. Sirivaidyapong, et al., (2001) established the fact that presence of prostatic fluid is generally avoided in cryopreservation of dog semen. Current study augmented that in vitro seminal parameters of spermatozoa in SP or three extenders during refrigeration (4℃) over four days (Rota et al., 1995). It expressed no specific effect of third fraction on the survival of dog sperm when egg yolk or a milk-based extender was added. The fractionated SRF was centrifuged and re-extended with autologous SP or three extenders (egg yolk-Tris, milk, or cream extender; all containing antibiotics). In addition, at this low temperature there is insufficient thermal energy for chemical reactions (Mazur, 1984). The presence of glucose and fructose in the diluents used for refrigeration can exert very important effects given the fact that metabolic routes have been found in both sugars, providing both different and complementing effects (Martínez & Rivera, 2022). Monosaccharides like glucose and fructose are quickly metabolized by sperm with finding that glucose is more rapidly consumed in comparison of fructose, but not all sugar and carbohydrates are used (Lisboa et al., 2021).

CONCLUSION

The main nutrients required for sperm metabolism are glucose and/or fructose. During preservation when semen is chilled or frozen the semen extender must provide protection against cold shock and damage. Egg yolk or low-density lipoproteins (LDL) are used to provide energy and prevent cold shock which effects cell membrane integrity. Last, antibiotics such as penicillin and streptomycin, or gentamycin will help to prevent microbial growth. The current study indicated that the concept of storage at low temperature is that the biological process of a living cell is lowered and at extremely low temp could reach to a point of suspended animation. Results show significantly improved sperm motility and acrosomal integrity in the fructose-based semen extender as compared to glucose-based semen extender. As a conclusion, addition of fructose in semen extender has positive influence on sperm quality.
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AUTHOR CONTRIBUTIONS

Muhammad Ali Raza: Writing-original draft, Conceptualization, conducted experiment. Muhammad Ali Raza: Conceived the Idea, overall management of the work. Usman Ahmad: Analysis, Interpretation of Results and Visualization. Kissa Zahra and Laraib Zahra Baloch: Analysis and Interpretation of Results and Review the literature. Muhammad Ali Raza: Data Collection and field experiment layout preparation. Murtaza Hussain Andrabi: Reviewed the original draft. All the authors reviewed the results and approved the final version of the manuscript.

COMPETING OF INTEREST

The authors declare no competing interests.

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