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Original Article

Effect of bovine and human follicular fluid on semen quality of fresh and frozen-thawed semen in dual purpose Simmental (Fleckvieh) bulls: a new method of adding follicular fluid to bull semen

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Abstract

Background: Follicular fluid (FF) is a biological fluid that contains many compounds such as proteins, hormones, metabolites, antioxidants, *etc*. **Aims:** The purpose of this research is to investigate the effects of adding cattle and human follicular fluid on bulls' semen quality during the freezing process. **Methods:** Semen sampling from 12 Simmental bulls was performed in a period of 3 months (36 ejaculations with three ejaculates per bull). Each ejaculate was divided into four equal portions. Three portions were used for control (C) (semen without follicular fluid), semen containing human follicular fluid (HFF), semen containing cow follicular fluid (CFF). Another part of semen was also used to prepare seminal plasma. Sperm quality assessment was performed on fresh and thawed frozen (immediately after thawing, 1 and 2 h after thawing) semen. **Results:** Adding human follicular fluid to the bulls' semen can slow down the process of reducing sperm motility and can delay the increase in the percentage of dead sperm in frozen-thawed semen. **Conclusion:** The human follicular fluid maintains the viability and motility of bull sperms better than the control and bovine follicular fluid. The possible effect of human follicular fluid on the metabolism of sperms during the process of freezing and thawing needs to be clarified in future studies.

Key words: Follicular fluid, Freezing, Semen quality, Simmental bulls

Introduction

Follicular fluid is the substance that accumulates in the extracellular space of follicles. This fluid mainly contains blood proteins from the theca layer's blood vessels. Secretions from the oocytes, granulosa cells and theca also contribute to the formation of the follicular fluid (Bianchi *et al*., 2016; Rusco *et al*., 2023). Human follicular fluid, a biological fluid, can be obtained easily and in large quantities without compromising oocyte quality during oocyte retrieval (Bianchi *et al*., 2016). The chemical composition of follicular fluid has been extensively studied in different domestic animals and humans (Caravaglios and Cilotti, 1957; David *et al*., 1973). The majority of plasma proteins, including albumin, can be found in follicular fluid (Edwards, 1974). Studies have demonstrated that albumin facilitates the capacitation process in sperms from rats, mice, and humans (Moubasher, 1986) by promoting cholesterol efflux from the membrane. Human follicular fluid, as well as that of pig and cow (Brantmeier *et al*., 1987), predominantly contains high-density lipoprotein (HDL) during laboratory incubation (Jaspard *et al*., 1997). HDL has been identified as a cholesterol receptor and a potential component of the cell membrane (Ehrenwald *et al*., 1990). Cholesterol, the primary sterol in most mammalian cell membranes (Go and Wolf, 1983), is predominantly located in the plasma membrane, accounting for 80 to 90% of total cellular cholesterol (Lange and Ramos, 1983). Membrane cholesterol plays a crucial role in membrane physical properties, including permeability and phase transition, due to its interactions with proteins and phospholipids. It contributes to the compactness and rigidity of the plasma membrane at the transition temperature (Vanderkooi *et al*., 1974). Consequently, a decrease in the ratio of cholesterol to phospholipids is linked to an increase in the fluidity and permeability of the cell membrane (Cooper *et al*., 1978). Research has demonstrated that cholesterol exchanges occur between the cell membrane, serum lipoproteins, and lipid rafts (Bruckdorfer *et al*., 1968). By modifying the cholesterol-to-phospholipid ratio, specifically by increasing phospholipid levels, the structure and function of the membrane can be affected when temperature

decreases (Parks *et al*., 1981). In the process of sperm capacitation, cholesterol is naturally removed from the sperm cell membrane through receptor molecules found in the extracellular environment of sperm, such as albumin and HDL, both of which are present in follicular fluid (Revelli *et al*., 2009). Consequently, the inclusion of follicular fluid in sperm prior to freezing is speculated to reduce spermatozoa membrane damage during the freezing process. On the other hand, steroid hormones, particularly progesterone, are abundantly found in follicular fluid (Baldi *et al*., 2009; Sun *et al*., 2017). Previous studies have indicated that the addition of follicular fluid can induce the acrosome reaction (Tesaăík, 1985; Calvo *et al*., 1989) and enhance sperm motility and activity (Mbizvo *et al*., 1990; Falcone *et al*., 1991). Furthermore, the progesterone found in follicular fluid can stimulate calcium increase in semen (Juneja *et al*., 1993) and oocyte maturation (Kadam and Koide, 1991). On the contrary, other studies have shown that the addition of follicular fluid to semen can actually hinder semen attachment to the zona pellucida in both pigs (Funahashi and Day, 1993) and humans (Yao *et al*., 1996). To our knowledge, the only available report regarding the impact of adding follicular fluid before freezing on semen and assessing frozen-thawed semen motility quality is the study conducted by Zeng *et al*. in 2001. They found that adding 75% volume of bovine follicular fluid to washed and centrifuged sperm (sperm without seminal plasma) from pigs 1 h prior to freezing could enhance forward motility and velocity of spermatozoa after thawing (Zeng *et al*., 2001). However, as of now, there are no existing studies that have investigated the effects of adding follicular fluid on the motility of bull semen during the freezing process. Therefore, the aim of this study is to evaluate effect of bovine and human follicular fluid on semen quality of fresh and frozen-thawed semen in dual purpose Simmental (Fleckvieh) bulls.

Materials and Methods

Animals

This study utilized 12 dual-purpose German Simmental bulls (Fleckvieh) from the Iran Simmental Cattle Breeding Center located in Amol, Mazandaran, Iran (Amarddam Tabarestan Co.). These bulls had previously undergone assessments of their reproductive potential and had received approval from the national breeding center. The bulls were chosen as they were healthy and fertile sires (aged between 3 and 6 years). All bulls at the center originated from a purebred Simmental breeding herd and were kept and fed under the same conditions. The specific details regarding the compositions, ingredient percentages, and feed quantities consumed by the animals can be found in Table 1.

Collection of human follicular fluid

Human follicular fluid was acquired from women undergoing *in vitro* fertilization (IVF) treatment and exhibited a high average number of oocytes. The use of these samples was with the full consent of the donors. These women also had male-factor infertility. Human monophasic gonadotropin was administered after the administration of buserelin to stimulate the ovaries. A sample of human follicular fluid, free from any blood contamination, was obtained from the largest follicles of individuals undergoing treatment. The follicular fluid underwent centrifugation at 300 g for 10 min to eliminate cellular debris. Subsequently, heat inactivation was performed by exposing the fluid to a temperature of 56°C in a warm water bath for 30 min, ensuring the complement system was inactivated. To achieve maximum sterilization, the obtained fluid was passed through 0.44 µm filters and initially cooled at 4°C before being stored at -20°C (Tesaăík, 1985).

Collection of cow follicular fluid

Fresh ovaries from sexually mature cows at the Amol County industrial slaughterhouse were colected. These ovaries were placed in vacuum flasks filled with normal saline adjusted to 37°C, supplemented with 1000 units of penicillin and 1 milligram per pl of streptomycin. Within 1 h of slaughter, the ovaries were transported to the laboratory of the Faculty of Veterinary Medicine at Amol University of Innovative Technologies for subsequent processing. The ovaries underwent four washes with normal saline enriched with penicillin and streptomycin to eliminate contaminants. Then they were washed in physiological serum and rinsed with 70% ethanol to eliminate surface contamination from the

Table 1: Composition, percentage, and amounts of the diet for dual-purpose Simmental German reproductive breed bulls (Fleckvieh)

Diet combinations	Amount	Chemical compounds					
	kg	Crude protein (%)	NDF (%)	ADF (%)	Fat (%)	Ash (%)	Dry material (%)
Concentrate	9	14.68	16.8	13.1	3.2	7.4	89.2
Silo	18	8.5	54.5	32.7	1.8	5.7	25
Alfalfa	3	16.8	44.7	34.6	2.5	9.7	1.88
Straw	*Ad libitum*	3.9	70.3	45.5	1.1	9.8	94.8
Mineral	*Ad libitum*	-	-	-	-	-	-
Water	*Ad libitum*	-	-	-	-	-	-

Supplement Calcium: 0.74%, Phosphorus: 0.53%, Sodium: 0.49%, Magnesium: 0.29%, Zinc: 375 ppm, Manganese: 44.381 ppm, Cobalt: 0.01 ppm, Selenium: 75.2 ppm, and Vitamin additives (vitamin A: 7500 IU/kg, vitamin D3: 1000 IU/kg, vitamin E: 10 mg/kg). agnesium: 1.2%, Sodium: 7%, Iron: 355 mg/kg, Zinc: 1560 mg/kg, Copper: 390 mg/kg, Manganese: 1560 mg/kg, Selenium: 5.7 mg/kg, Cobalt: 3 mg/kg, Iodine: 5.15 mg/kg. Source: The data were calculated by the Animal Nutrition Laboratory, Faculty of Veterinary Medicine, University of Tehran

ovaries. The follicular fluid was extracted by aspirating large antral follicles, measuring 10 to 20 ml in diameter, from ovaries that had undergone corpus luteum regression. A sterile 5-ml syringe (non heparinized) fitted with an 18-gauge needle was used. Follicles with a reddish or turbid appearance were excluded from the fluid collection. The aspirated follicular fluid was placed in Eppendorf straws and centrifuged at 300 g for 10 min at 5°C to separate granulosa cells, blood cells, oocytes, and residual tissue fragments. The resulting supernatant fluid was then heated at 56°C for 30 min in a warm water bath and subsequently stored at a temperature of -20°C until it was ready to be used.

Semen collection and addition of follicular fluid

Semen collection was carried out for a duration of three months, from October to November 2023, obtaining a total of 36 ejaculates (three ejaculates per bull). To collect semen samples from each bull, an artificial vagina heated to a temperature of 46°C was utilized (with three repetitions for each bull). Before collection, the bull underwent sexual preparation involving three pseudo mounts, where it stood for 10 min next to a restrained dummy in the semen collection box. Immediately following semen collection, semen volume and sperm concentration were assessed. The sperm concentration was measured using an SDM 1 photometer (Minitube, Tiefenbach, Germany). Each ejaculate was then divided into four equal portions. Three portions were used for control (C) (semen without follicular fluid), semen containing human follicular fluid (HFF), semen containing cow follicular fluid (CFF). Another part of semen was also used to prepare seminal plasma. The fourth portion was used to prepare seminal plasma in order to add it to the semen of the C group to reach the concentration of this group to 750 million sperm per ml. For this purpose, 1 ml of this portion was separated and centrifuged for 10 min at 3000 rpm. Subsequently, recovered seminal plasma was re-centrifuged to diminish the remaining cells.

In this study, we did not want to wash bovine sperm (as centrifugation can damage the semen and affect their ability to be frozen), therefore, follicular fluid was added to the different ejaculates of each bull in a manner that ensures every ejaculation receives the same amount of follicular fluid. This is important for accurately assessing the impact of adding a consistent amount of follicular fluid on sperm quality parameters, considering that each ejaculation has varying concentrations of spermatozoa. To achieve this, a source with a desirable concentration of one ejaculate for freezing, ranging from 750 million to 1 billion spermatozoa per ml of seminal fluid, is required (Falcone *et al*., 1991). Therefore, we chose a concentration of 750 million sperm per ml as the benchmark and ejaculates with a concentration exceeding 800 million spermatozoa per ejaculate were analyzed. Therefore, according to the stated content, we decided to invent a more accurate method. In this method, semen concentration was calculated at the beginning after semen collection and a concentration of 750 million sperm per ml has been chosen as the benchmark. This means that ejaculates with a concentration exceeding 800 million spermatozoa per ejaculate will be analyzed. The amount of follicular fluid needed for each ejaculation to reach a population of 800 million spermatozoa per ml was calculated by the below formula:

$$[a \times (b + c) = (a \times b) + (a \times c)]$$

For the control sample, which will not contain follicular fluid, the seminal plasma of the same bull was added to the same ejaculation to reach the concentration of 800 million spermatozoa per ml.

Semen cryopreservation

Collected semen samples were preserved by freezing them using a one-step dilution method. Triladyl (Minitube, Tiefenbach, Germany), a semi-prepared extender, was used along with water and egg yolk in specific ratios. The process involved pouring a complete package of Triladyl (250 g) into a graduated flask and gradually adding 750 ml of deionized water. This mixture was referred to as the stock solution. Pure egg yolk (250 ml) was added to the stock solution. The eggs were cleaned, dried, and disinfected by passing them through an alcohol flame. The yolk and egg white were separated using an egg yolk separator tool, and the yolk was filtered to remove any remaining white. The stock solution was slowly added to the yolk while stirring with a magnetic stirrer. Finally, the diluent was filtered with sterile gas.

To create the pre-extender dilution mixture, the prepared diluent was gently added to the semen in a 1:1 ratio. This mixture was then placed in a water bath set at 34°C for 10 min.

The required final volume of diluent was calculated using the following formula:

Number of semen doses = (volume of semen × sperm liquid concentration × progressive motility percentage of sperm × percentage of natural sperm morphology) ÷ (number of sperms per straw [15 million]) (Tahmasbian *et al*., 2022).

The final solution was prepared by combining the pre-extender dilution solution with the necessary amount of diluent. The resulting flasks were kept at room temperature for 15 min. Subsequently, the 0.5 ml straws (Minitube, Slovakia) were filled and sealed using an automatic machine called MPP Uno (Minitube, Tiefenbach, Germany) at the laboratory packaging station. The filled straws were then placed on trays and stored in a refrigerator set at 4°C for 3 h to reach equilibrium. After the equilibration period, the packaged straws were frozen using an MT Freezer device (Minitube, Tiefenbach, Germany). The freezing protocol involved a gradual decrease in temperature from +4°C to -12°C at a rate of -4°C per min, followed by a decrease from -12°C to -40°C at a rate of -40°C per min, and finally, a decrease from -40°C to -140°C at a rate of -50°C per min. The frozen straws were stored individually in canisters inside a liquid nitrogen

container.

Examination of semen motility

The assessment of semen motility on both fresh and frozen-thawed was performed by using the Computer-Assisted Sperm Analysis (CASA) software (Intelligent Technology, Tehran, Iran). To thaw the frozen semen, the straws were placed in 37°C water for 40 s. Various sperm motility parameters were evaluated, including progressive motility (PM), curvilinear velocity (VCL), which represents the actual velocity of sperm along its curvilinear path, straight-line velocity (VSL), average path velocity (VAP), mean angular displacement (MAD), lateral head displacement (ALH), beat cross frequency (BCF), linearity (LIN) which is the ratio of VSL to VCL, wobble (WOB) which is the ratio of VAP to VCL, and straightness (STR) which is the ratio of VSL to VAP. All analyses were performed using a light microscope equipped with a heated stage to maintain the temperature of samples at 37°C and included a chamber to prevent sperm motility decline during analysis (sperm meter, depth 10-µm, surface graticule, 100x 0.1 SQMM).

Viability and morphological examination of semen

To evaluate the proportion of viable spermatozoa in each ejaculation, Eosin-Nigrosin staining (2% Eosin Y, 0.025/15405 - 4% Nigrosin, 0.029/15405, Minitube, Tiefenbach, Germany) was employed. The process can be summarized as follows: following the introduction of sperm into the final diluent, a mixture of sperm and the final diluent was gently combined with a drop of 2% Eosin and two drops of 4% Nigrosin using a pipette. This mixture was then spread on a glass slide to form a smear. The prepared smears were allowed to air-dry on a warm plate, after which a minimum of 200 sperm were examined under a microscope. Dead sperm, possessing high cell membrane permeability, absorbed the Eosin stain and exhibited a red coloration, whereas viable sperm, which did not absorb this stain, remained completely distinct. The Eosin-Nigrosin staining technique was also employed to evaluate the percentage of abnormal spermatozoa before and after freezing. At least 200 sperm in each slide were examined for abnormalities in the head, midpiece, and tail, as well as the presence of cytoplasmic droplets.

Evaluation of sperm membrane integrity

The health of sperm membranes was evaluated using the hypo osmotic swelling test (HOST). To perform the test, 10 µL of both fresh and thawed semen were mixed with 100 µL of a hypoosmotic medium containing fructose (9 g per L) and sodium citrate (4.9 g per L). The mixture was then incubated at room temperature for 30 min. Subsequently, at least 3 drops of the incubated sample were examined using a phase-contrast microscope. The microscopic examination was conducted on a preheated glass slide maintained at a temperature of 37°C and at a magnification of ×400. A minimum of 200 sperms were counted on each slide, and the percentage of sperms with intact membranes was calculated (Ijab *et al*., 2022).

Statistical analysis

At first, the normality of the data related to the quality parameters of fresh and frozen-thawed semen (0, 1, 2 h) was evaluated by the Shapiro-Wilk test. Quality parameters of sperm (15 indexes) were analyzed by two-way analysis of variance (ANOVA) on data related to fresh and frozen-thawed sperms, treatment groups (type of follicular fluid), and fresh and frozen-thawed sperms × treatment groups interaction. Significant differences between means were determined using Tukey's post hoc test. The statistical analysis was performed using the SPSS ver. 26 software (SPSS Inc., Chicago, IL, USA) and the graphs were prepared using GraphPad Prism software ver. 9 (GraphPad Software, La Jolla, CA, USA). All results were expressed as mean±SD. For all analyses, $P<0.05$ was considered as the level of significance.

Results

Sperm parameters (15 indexes) in fresh and frozen-thawed semen containing different follicular fluids at different time points are shown in Table 2.

Progressive motility and viability of sperms in the group that contained human follicular fluid were significantly higher than the control and the group that contained animal follicular fluid ($P<0.05$). A higher percentage is observed when human follicular fluid is used. In terms of other investigated indexes, there were no significant differences between the treatments.

As a result, based on the two-way ANOVA analysis, the effect of treatment groups in the increase of the progressive motility of sperms was significant ($F2.33=10.39$, $P<0.001$), and the effect of the fresh and frozen-thawed semen at different time points was also significant ($F3.99=192.42$, $P<0.001$), and the interaction effect between group and type of semen was not significant ($F6.99=0.09$, $P=0.99$). Also, the effect of treatment groups in the increase of the viability of sperms was significant ($F2.33=14.43$, $P<0.001$), and the effect of the fresh and frozen-thawed semen at different times was also significant ($F3.99=60.07$, $P<0.001$), and the interaction effect between group and type of semen was not significant ($F6.99=0.33$, $P=0.91$).

Discussion

This research aimed to examine how human and bovine follicular fluid impact the semen quality of dual-purpose Simmental (Fleckvieh) bulls, before and after freezing. Prior investigations have indicated that the inclusion of follicular fluid promotes the acrosome reaction (Kadam and Koide, 1991; Juneja *et al*., 1993; Yao *et al*., 1996) and improves semen motility and activity (Funahashi and Day, 1993; Wen-Xian *et al*., 2001; Hong *et al*., 2003). Moreover, the presence of progesterone in follicular fluid can increase the calcium

Table 2: Comparison of sperm quality parameters in fresh and frozen-thawed sperms (0, 1, 2 h) containing different follicular fluids. Data are presented as the mean±SD (n=12 in each group)

Sperm quality parameter	Type of follicular fluid	Fresh semen	Frozen-thawed semen		
			Hour 0	Hour 1	Hour 2
PM (%)	C	$60.71 \pm 8.56^{a, B}$	$44.82 \pm 11.01^{b, B}$	$33.92 \pm 9.85^{c, B}$	$23.43 \pm 8.53^{d, B}$
	HFF	$70.72 \pm 8.74^{a, A}$	$55.89 \pm 11.37^{b, A}$	$45.31 \pm 9.08^{c, A}$	$35.12 \pm 7.66^{d, A}$
	CFF	$60.87 \pm 10.26^{a, B}$	$43.55 \pm 8.23^{b, B}$	$34.63 \pm 7.55^{c, B}$	$23.88 \pm 5.29^{d, B}$
VCL (µm/S)	C	44.53 ± 9.48^{a}	28.38 ± 8.82^{b}	22.11 ± 8.28^{c}	14.98 ± 8.61^{d}
	HFF	45.54 ± 15.88^{a}	30.64 ± 10.37^{b}	20.80 ± 5.48^{c}	14.64 ± 5.23^{d}
	CFF	40.98 ± 14.04^{a}	26.76 ± 7.70^{b}	22.36 ± 8.48^{b}	16.90 ± 7.17^{c}
VSL (µm/S)	C	23.50 ± 7.50^{a}	13.31 ± 5.87^{b}	10.89 ± 5.13^{b}	6.52 ± 4.90^{c}
	HFF	24.23 ± 9.48^{a}	16.30 ± 5.80^{b}	10.50 ± 2.29^{c}	7.00 ± 4.57^{d}
	CFF	21.83 ± 8.27^{a}	13.63 ± 4.35^{b}	10.35 ± 4.07^{b}	6.98 ± 3.22^{c}
VAP (µm/S)	C	29.35 ± 8.10^{a}	17.29 ± 6.18^{b}	13.84 ± 5.67^{b}	8.60 ± 5.59^{c}
	HFF	29.83 ± 10.93^{a}	20.56 ± 7.10^{b}	13.23 ± 2.85^{c}	8.34 ± 3.23^{d}
	CFF	27.27 ± 9.82^{a}	17.21 ± 4.69^{b}	13.46 ± 5.25^{b}	9.55 ± 4.18^{c}
MAD	C	37.002 ± 7.73^{a}	24.01 ± 12.01^{b}	19.43 ± 10.78^{b}	14.31 ± 9.58^{c}
	HFF	38.56 ± 12.83^{a}	21.97 ± 10.10^{b}	15.16 ± 9.01^{c}	12.04 ± 6.94^{c}
	CFF	34.58 ± 13.16^{a}	22.06 ± 8.44^{b}	18.76 ± 8.30^{b}	13.89 ± 8.16^{c}
ALH (µm)	C	2.31 ± 0.35^{a}	1.66 ± 0.40^{b}	1.27 ± 0.38^{b}	0.91 ± 0.40^{c}
	HFF	2.24 ± 0.68^{a}	1.72 ± 0.53^{a}	1.23 ± 0.34^{b}	0.92 ± 0.32^{c}
	CFF	2.09 ± 0.63^{a}	1.50 ± 0.47^{b}	1.34 ± 0.47^{b}	1.08 ± 0.44^{c}
BCF (Hz)	C	3.87 ± 0.70^{a}	2.22 ± 1.28^{b}	1.76 ± 1.12^{b}	1.04 ± 0.81^{c}
	HFF	3.66 ± 0.59^{a}	2.43 ± 1.39^{b}	1.26 ± 0.85^{c}	0.88 ± 0.53^{c}
	CFF	3.68 ± 1.01^{a}	2.05 ± 0.81^{b}	1.53 ± 0.82^{b}	0.98 ± 0.74^{c}
LIN (%)	C	38.55 ± 7.94^{a}	27.61 ± 7.69^{b}	22.28 ± 7.50^{c}	14.75 ± 7.31^{d}
	HFF	37.69 ± 8.29^{a}	31.38 ± 7.58^{a}	22.68 ± 6.99^{b}	14.95 ± 4.15^{c}
	CFF	36.93 ± 8.98^{a}	27.33 ± 5.92^{b}	22.16 ± 5.97^{c}	16.15 ± 6.29^{d}
Viab (%)	C	$75.86 \pm 4.08^{a, B}$	$67.40 \pm 14.10^{ab, B}$	$64.26 \pm 10.25^{b, B}$	$51.92 \pm 9.91^{c, B}$
	HFF	$85.78 \pm 4.99^{a, A}$	$79.14 \pm 4.81^{b, A}$	$73.61 \pm 9.21^{b, A}$	$66.02 \pm 9.63^{c, A}$
	CFF	$76.85 \pm 6.57^{a, B}$	$68.80 \pm 4.59^{b, B}$	$63.11 \pm 9.83^{c, B}$	$53.20 \pm 7.34^{c, B}$
Morph (%)	C	10.56 ± 3.93	7.91 ± 3.93	7.82 ± 3.73	9.32 ± 3.49
	HFF	10.58 ± 7.39	9.03 ± 5.75	9.08 ± 6.21	9.08 ± 4.92
	CFF	12.84 ± 7.06	11.26 ± 7.43	10.29 ± 5.54	10.15 ± 3.81
Head (%)	C	2.31 ± 2.22	1.77 ± 1.62	1.86 ± 1.69	2.52 ± 2.23
	HFF	1.82 ± 1.73	1.59 ± 1.43	1.73 ± 1.31	2.04 ± 1.97
	CFF	2.15 ± 1.68	1.87 ± 1.27	1.50 ± 1.12	1.80 ± 1.45
Mid. P (%)	C	1.28 ± 1.12	2.32 ± 1.25	1.32 ± 1.11	0.71 ± 0.54
	HFF	1.56 ± 1.23	1.68 ± 1.44	0.88 ± 0.64	0.80 ± 0.51
	CFF	1.56 ± 1.35	0.84 ± 0.49	1.26 ± 1.33	1.64 ± 1.12
Tail (%)	C	6.76 ± 3.32	3.57 ± 2.30	4.30 ± 2.87	5.76 ± 2.65
	HFF	6.97 ± 6.83	5.76 ± 4.82	6.35 ± 5.71	6.05 ± 3.78
	CFF	8.76 ± 4.67	8.54 ± 7.31	7.52 ± 4.79	6.70 ± 4.08
Cy.D (%)	C	0.20 ± 0.14	0.24 ± 0.11	0.32 ± 0.16	0.32 ± 0.19
	HFF	0.20 ± 0.13	0	0.11 ± 0.06	0.19 ± 0.12
	CFF	0.37 ± 0.15	0	0	0
HOST (%)	C	50.50 ± 13.80^{a}	48.13 ± 8.83^{a}	40.93 ± 9.57^{b}	35.82 ± 10.42^{b}
	HFF	55.61 ± 12.88^{a}	43.02 ± 8.50^{b}	37.42 ± 8.15^{c}	35.17 ± 9.86^{c}
	CFF	56.96 ± 15.83^{a}	38.68 ± 13.29^{b}	35.39 ± 11.55^{b}	30.19 ± 13.36^{c}

[a, d] Different superscript small letters in the same row indicate a significant difference between fresh and frozen (0, 1, 2 h) sperms in each group ($P<0.05$), values with different superscript capital letters ([A, B]) in a column indicate a significant difference between treatments (type of follicular fluids) at each time point ($P<0.05$), and values with no superscript letters are not significantly different ($P>0.05$). The letters represent significant differences between groups in each index of sperm separately. C: Control, HFF: Human follicular fluid, CFF: Cow follicular fluid, PM: Progressive motility, VCL: Curvilinear velocity of frozen-thawed semen, VSL: Straight line velocity of frozen-thawed semen, VAP: Average path velocity of frozen-thawed semen, MAD: Malondialdehyde, ALH: Lateral head displacement, BCF: Beat cross-frequency, LIN: linearity, Viab: Viability, Morph: Abnormal morphology, Head: Head abnormality, Mid. P: Mid-piece abnormality, Cy.D: Cytoplasmic droplet, Tail: Tail abnormality, and HOST: Hypo osmotic swelling test

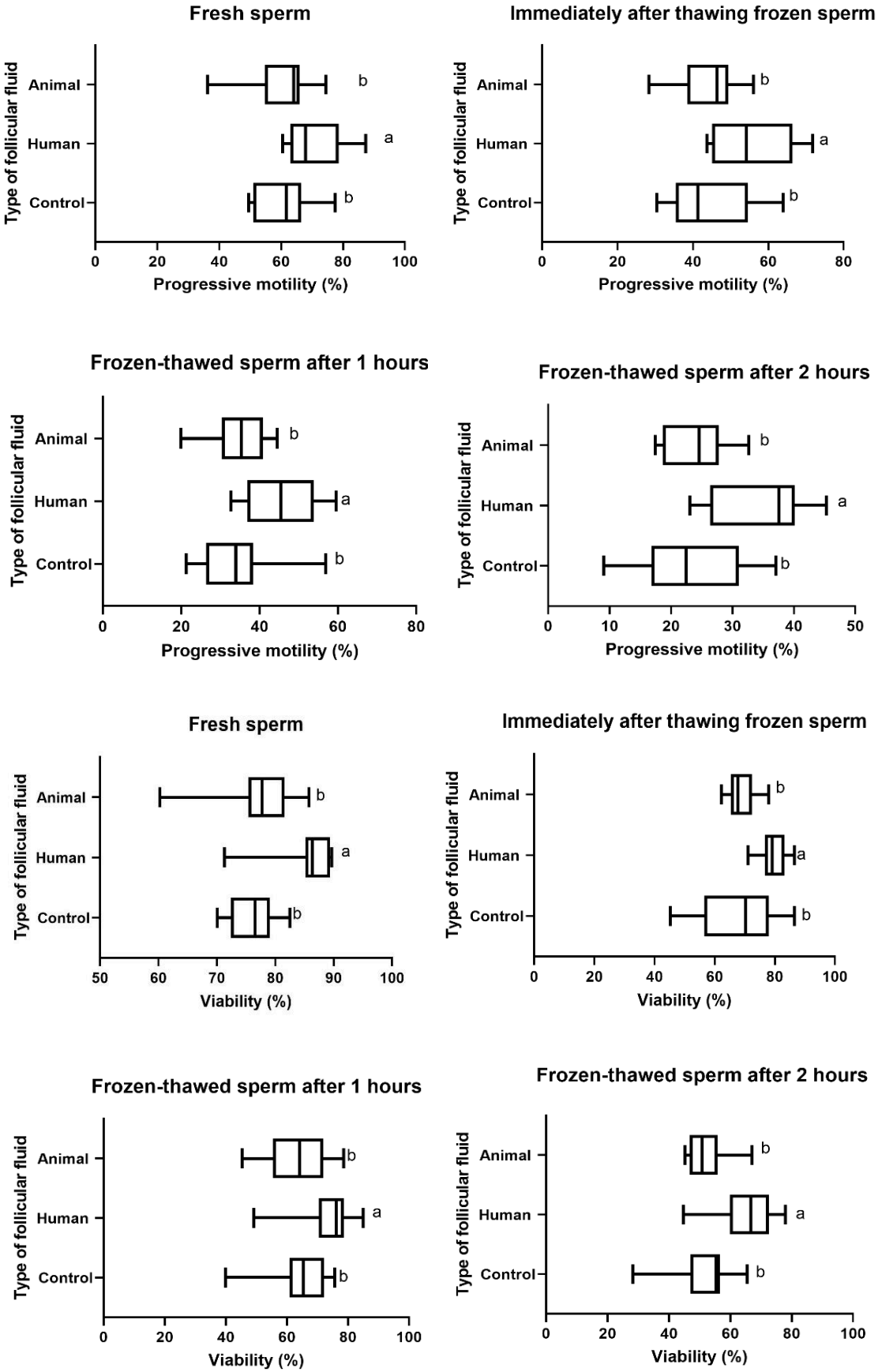

Fig. 1: Boxplots displaying the difference between the average of progressive motility and viability in three treatments in fresh and freezing sperms. [a, b] Different letters indicate a significant difference among groups in each time point (P<0.05)

levels in sperm (Kadam and Koide, 1991) and stimulate oocyte maturation (Yeung *et al*., 2009).

Various authors have commented on the merits of human and animal follicular fluid impacts on the semen quality (Dorado-Silva *et al*., 2020; Liu *et al*., 2022), For example, Getpook and Wirotkarun (2007) examined sperm motility stimulation and preservation with various concentrations of follicular fluid. The study showed that, follicular fluid at 20-50% concentrations can stimulate active spermatozoa to become more rapidly progressive spermatozoa and maintained its effect for up to 12 h (Getpook and Wirotkarun, 2007). In another study Avrech *et al*. (1997) examined acrosomal status of human spermatozoa after follicular fluid in relation to semen parameters and fertilizing capacity *in vitro*, the results showed that, the effect of follicular fluid on the acrosomal status correlated positively with the effect obtained by the calcium ionophore. The results of our research revealed that adding human follicular fluid to the semen of cattle, both in fresh sperm and during equilibration, not only had no effect on sperm motility but also led to a slight reduction in progressive motility of spermatozoa, which was not statistically significant. In another study Huang *et al*. (2002) examined Biochemical compositions of follicular fluid and the effects of culture conditions on the *in vitro* development of pig oocytes, these authors found that, neither the fresh *in vitro* maturation (IVM) nor filtered IVM-conditioned medium has positive effect on the *in vitro* development (IVD) of oocytes. Coculture with cumulus cell monolayers and the stepwise medium replacement procedures (SMRP) were not beneficial to the development of *in vitro* fertilization (IVF) pig oocytes (Huang *et al*., 2002). Zhou *et al*. (2004) examined effect of extenders and temperatures on sperm viability and fertilizing capacity of boar semen during long-term liquid storage. The results of the study showed that, the Zorlesco diluent with Polyvinyl alcohol (PVA) substituting bovine serum albumin (BSA) maintained sperm viability and fertilizing capacity after 8 days of semen storage (Zhou *et al*., 2004). In our study, an interesting finding was that semen frozen-thawed samples containing human follicular fluid showed higher motility compared to samples without follicular fluid. Esmaeilpour *et al*. (2014) examined effect of follicular fluid and platelet-activating factor (PAF) on lactate dehydrogenase c expression in human asthenozoo-spermic samples, the results showed that, PAF had more beneficial effects than fulicular fluid on sperm motility in the asthenozoospermic samples. Moreover, Cruz *et al*. (2014) evaluated the influence of follicular fluid added to the maturation medium on the quality of bovine embryos produced *in vitro*. Follicular fluid slowed the meiotic progression and migration of cortical granules (CG) and contributed to increases in inner cell mass number (Cruz *et al*., 2014). On the other hand, Bahmanpour *et al*. (2012) examined the effect of the follicular fluid on sperm chromatin quality in comparison with conventional media. They found that, incubation of sperm in the follicular fluid increases sperms with normal histone, normal chromatin protamine and sperm with normal head size (Bahmanpour *et al*., 2012). Huyser *et al*. (1997) examined the influence of male, female, and fetal cord sera, follicular fluid, and seminal plasma on human sperm-zona pellucida binding, using the hemizona assay. They reported that sperm-zona pellucida binding was enhanced by 10% concentration follicular fluid, whereas variable results were obtained with addition of sera (Huyser *et al*., 1997). Briton-Jones *et al*. (2001) examined the effects of follicular fluid and PAF on motion characteristics of poor-quality cryopreserved human sperm. They showed that incubation in follicular fluid increases overall motility and the percentage of sperm with fast progressive motility in normospermic but not oligospermic samples. Incubation with PAF increased overall motility and the percentage of sperm showing nonprogressive motility in both oligospermic and normospermic samples (Briton-Jones *et al*., 2001).

It should be noted that in this study, a novel approach was used for the first time to add follicular fluid into semen. Previous studies investigating the impact of follicular fluid on sperm quality involved adding it to semen at varying ratios of seminal fluid volume, ranging from 10% to 75% (Calvo *et al*., 1989). This was done either after separating seminal plasma from spermatozoa through washing, where follicular fluid was added to sperm without seminal plasma, or by adding follicular fluid to unwashed seminal fluid without considering the sperm count in each ejaculation (Mbizvo *et al*., 1990).

In conclusion, adding human follicular fluid to the bulls' semen can slow down the process of reducing sperm motility and can delay the increase in the percentage of dead sperm in frozen-thawed semen.

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Conflict of interest

All authors declared no conflict of interest.

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