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RESEARCH ARTICLE

Effect of Different Extenders on the Sperm Quality Parameters of Hu Ram Semen Preserved at 16°C

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Abstract

The purpose of this experiment was to determine the effects of different extenders on the sperm quality parameters of Hu ram semen preserved at 16°C. The quality parameters studied include total sperm motility, progressive motility, survival time, plasma membrane integrity, acrosome integrity and some kinematic parameters, such as the curvilinear velocity. Each ram ejaculated approximately twenty times and ejaculates were collected every two days interval during breeding season. Three Hu rams were used in experiments. The ejaculates were pooled and diluted (1:10) with extenders A (Tris-Fructose based), B (Fructose-Sodium Citrate based), C (Glucose based), D (Fructose based) and E (Control, Physiological saline solution) and then stored at 16°C. The above parameters were detected every 24 h. The total sperm motility, progressive motility, acrosome integrity and some kinematic parameters of extender A were the highest compared to those of other extenders and decreased slowly within 24 to 144 h. The effective survival time of sperm preserved in extender A was 74.50±4.82 h, and the total survival time was 412.67±2.52 h, which was significantly higher than those of the other four extenders ($P \leq 0.05$). The acrosome integrity of extender A was the highest within 24 to 144 h and significantly higher than those of the other extenders within 48 to 144 h ($P \leq 0.05$). Compared with the other extenders, extender A had numerically the highest plasma membrane integrity within 24 to 96 h of preservation ($P > 0.05$). In conclusion, extender A improved the sperm quality of Hu ram semen, which could be used for artificial insemination for up to 144 h of preservation.

Keywords: *Hu Ram, 16°C, Semen extender, Semen quality*

Farklı Sperm Sulandırıcılarının 16°C'de Saklanan Hu Koç Sperm Kalitesi Parametreleri Üzerine Etkisi

Öz

Bu çalışmanın amacı, farklı sperm sulandırıcılarının 16°C'de saklanan Hu koç sperm kalitesi parametreleri üzerindeki etkilerini belirlemekti. İncelenen kalite parametreleri arasında toplam sperm motilitesi, progresif motilite, canlı kalma süresi, plazma membran bütünlüğü, akrozom bütünlüğü ve eğrisel hız gibi bazı kinematik parametreler bulunuyordu. Her koçtan yaklaşık yirmi kez sperm alındı ve üreme mevsimi boyunca koçlardan iki günde bir ejekülat toplandı. Deneylerde üç Hu koçu kullanıldı. Ejekülatlar havuz oluşturulduktan sonra, 1:10 oranında sırasıyla A (Tris-Fruktoz bazlı), B (Fruktoz-Sodyum Sitrat bazlı), C (Glukoz bazlı), D (Fruktoz bazlı) ve E (Kontrol, Fizyolojik tuzlu su) sulandırıcıları ile seyreltildi ve sonra 16°C'de muhafaza edildi. Adı geçen parametreler her 24 saatte bir tespit edildi. A sulandırıcısının kullanıldığı gruptaki toplam sperm motilitesi, progresif motilite, akrozom bütünlüğü ve bazı kinematik parametreleri diğer sulandırıcılara oranla en yüksek saptandı ve bu özellikler 24 ile 144 saat içinde yavaş yavaş azaldı. A sulandırıcısının kullanıldığı gruptaki spermlerin etkin canlılık süresi 74.50±4.82 saat ve toplam canlılık süresi 412.67±2.52 saat iken, bu değerler diğer dört sulandırıcıdan çok daha yüksek saptandı ($P \leq 0.05$). A sulandırıcısının kullanıldığı gruptaki spermlerin akrozom bütünlüğü 24 ile 144 saat içinde en yüksekti ve bu değerler diğer sulandırıcıların 48 ile 144 saat içinde sahip oldukları değerlerden çok daha yüksekti ($P \leq 0.05$). Diğer sulandırıcılarla karşılaştırıldığında A sulandırıcısının, 24 ile 96 saatlik sperm muhafazasında sayısal olarak en yüksek plazma membran bütünlüğüne sahip olduğu saptandı ($P > 0.05$). Sonuç olarak, A sperm sulandırıcısının, suni tohumlama için kullanılmak üzere 144 saate kadar muhafaza süresince sperm kalitesini artırdığı belirlendi.

Anahtar sözcükler: *Hu koçu, 16°C, Sperm sulandırıcısı, Sperm kalitesi*

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Introduction

Hu ram is a world-famous prolific sheep breed that has the advantages of two births a year and rapid growth and development; thus, the breeding scale of Hu ram is expanding rapidly [1]. Artificial insemination is a basic technology in livestock reproduction [2], which can make full use of the semen of superior male animals [3]. It is an important means to improve the reproductive efficiency and productivity of livestock [4].

Artificial insemination technology combined with the application of estrus synchronization technology can reduce breeding costs, prevent the spread of diseases, and achieve large-scale feeding and management [5]. Semen preservation is a key link in artificial insemination [6]. Semen preservation is usually divided into 16°C preservation, 4°C preservation and cryopreservation [7]. Owing to the presence of large amounts of polyunsaturated fatty acids (PUFA) in ram sperm plasma membranes and the absence of a robust antioxidant system in ram sperm and seminal plasma, ram sperm is highly sensitive to cryopreservation [8-10], which limits semen cryopreservation in sheep [11,12]. In production, semen is mostly preserved at 16°C, which has the advantages of simple operation and suitability for popularization and application, without special temperature control and refrigeration equipment; it also has practical application value.

At present, 4°C preservation and cryopreservation are the research focus of ram semen preservation [13]. There are relatively few reports on the preservation of ram semen at 16°C, and there are even fewer extender formulas described. However, most of the semen preserved at 16°C is used in production. This experiment is based on the extender used for 4°C or cryopreservation of ram semen. Extenders with better preservation were screened. Substances such as cryoprotectants were removed so as not to adversely affect semen preserved at 16°C. In the experiment evaluating the preservation of ram semen at 16°C, an excellent extender formula was selected. Arando et al.[14] indicated that OVIX was the best commercial extender for preservation of ram semen at 16°C. Total sperm motility preserved in the extender decreased to 40% in 96 h, and sperm progressive motility decreased to 20% in 96 h. Although the commercial extender has a good preservation effect, it is still far from meeting the needs of production. To prolong the storage time of semen at 16°C, therefore, extenders A (Tris-Fructose based), B (Fructose-Sodium Citrate based), C (Glucose based), D (Fructose based) and E (Control, Physiological saline solution) were used to preserve ram semen at 16°C in this experiment. The experiment objectively evaluated and screened an extender formula with the best preservation effect of ram semen at 16°C.

Material and Methods

Animals and Semen Collection

Three 2-to 3-years-old male Hu sheep having Body condition score (BCS) ≥3 (scale1-5) kept in the experimental sheep farm were used in this study. They were fed 0.2 kg concentrate/once, twice a day, and ad libitum hay and water. The experiment trimmed the ram abdomen and wash the foreskin with saline. A total number of 60 ejaculates were collected from the rams every two days interval in the morning by the artificial vaginal (AV) between October and December 2019. The water temperature in the AV was kept at 40~42°C. Ensure a certain degree of lubrication and pressure in the AV. It was brought back to the laboratory at 37°C within 20 min. The semen volume of each ram collected was about 1.5 mL, which was milky white and had no abnormal smell. The quality assessment was carried out quickly and only the total motility >80% and the morphologically abnormal sperm <15% were accepted. The ejaculates collected from the three rams were pooled and processed to eliminate variability. Semen sampling procedure was approved by the Animal care committee of the Yangzhou University.

Preparation of Semen Extender

Five different extenders, A (Tris-Fructose based), B (Fructose-Sodium Citrate based), C (Glucose based), D (Fructose based) and E (Control, Physiological saline solution), were prepared according to the ingredients and doses shown in *Table 1*.

Sperm Quality Evaluation

Total sperm motility, progressive motility and kinematic parameters were measured by CASA. Plasma membrane integrity was detected by HOST [15]. Acrosome integrity was detected by Coomassie brilliant blue staining [16]. The time when the sperm progressive motility is above 60% is called the effective survival time; when total sperm motility drops to zero, the time when all sperm die is called the total survival time [17].

Statistical Analysis of Data

All the test data were analyzed by SPSS25.0 statistical software. All results were expressed as mean values±SD. When the P value was significant (P≤0.05), Duncan's multiple range tests by ANOVA procedure were used to compare the mean value of the total sperm motility, progressive motility, kinematic parameters, plasma membrane integrity and acrosome integrity.

Results

Effects of Different Extenders on Total Sperm Motility

As seen in *Table 2,* the total sperm motility decreased with the extension of storage time when five kinds of extenders were used to preserve Hu ram semen at 16°C. Among them, the total sperm motility of extender A decreased steadily and slowly. Within 24 to 96 h of preservation, the total motility of sperm preserved in extender A was significantly

Table1. *Five different extender formulations*

Composition	A	B	C	D	E (Control)
Tris/g	15.3500	-	-	-	-
Citric acid/g	8.2000	-	-	-	-
Fructose/g	10.0000	7.2500	1.5310	9.0000	-
Glucose/g	-	5.7500	22.2490	1.0000	-
Sodium citrate/g	-	5.8500	-	-	-
Sodium bicarbonate/g	-	0.6250	-	0.5000	-
Polyvinyl alcohol/g	-	1.2500	-	-	-
EDTA/g	-	1.1500	-	-	-
Disodium hydrogen phosphate/g	-	-	0.7090	-	-
Sodium dihydrogen phosphate/g	-	-	0.5990	-	-
Sodium chloride/g	-	-	1.4310	0.3350	4.5000
Potassium chloride/g	-	-	0.1860	-	-
Calcium chloride dihydrate/g	-	-	-	0.0300	-
Magnesium chloride hexahydrate/g	-	-	-	0.0400	-
Penicillin sodium/g	0.1559	0.1559	0.1559	0.1559	-
Streptomycin sulfate/g	0.3472	0.3472	0.3472	0.3472	-
Sterilized ultra-pure water/mL	500.0000	500.0000	500.0000	500.0000	500.0000
PH	7.47	7.22	7.51	8.20	7.17

Table 2. *Effects of different extenders on the total motility of preserved sperm (mean±SD); %*

Storage Time	A	B	C	D	E (Control)
0 h	84.39±5.02^{ab}	89.18±6.41^{a}	81.19±1.72^{ab}	81.84±4.03^{ab}	84.53±4.95^{ab}
24 h	82.99±4.06^{a}	62.67±2.31^{b}	9.28±5.06^{e}	44.46±2.76^{c}	1.12±0.22^{f}
48 h	79.53±1.30^{a}	55.46±1.60^{b}	0.69±0.22^{e}	11.28±2.42^{d}	-
72 h	72.24±5.49^{a}	48.73±7.48^{b}	-	12.06±2.27^{c}	-
96 h	53.23±9.75^{a}	29.57±8.55^{b}	-	8.66±3.03^{c}	-
120 h	29.34±9.01^{a}	11.23±6.72^{bc}	-	1.77±0.33^{c}	-
144 h	6.04±3.53^{ab}	1.48±1.51^{bc}	-	0.48±0.23^{c}	-

$^{a\text{-}f}$ Values within the same row with different letters differ significantly (P≤0.05); - means that the sperm have all died, losing the significance of detection

higher than those in the other four extenders (P≤0.05). At 120 h of preservation, the total sperm motility of extender A was significantly higher than those of the other four extenders (P≤0.05). At 144 h of preservation, the total sperm motility in extender A was the highest and significantly higher than that in extender D (P≤0.05), but there was no significant difference between extender A and extender B (P>0.05).

Effects of Different Extenders on Sperm Progressive Motility

As seen in *Table 3,* the sperm progressive motility decreased with the extension of storage time when Hu ram semen was preserved with the five kinds of extenders at 16°C. Among them, the sperm progressive motility preserved by extender A decreased steadily and slowly. Within 24 to 96 h of preservation, the sperm progressive motility of extender A was significantly higher than those of the other four extenders (P≤0.05). At 120 h of preservation, the sperm progressive motility of extender A was significantly higher than those of the other four extenders (P≤0.05). At 144 h of preservation, the sperm progressive motility of extender A was the highest, but the difference was not significant (P>0.05).

Effects of Different Extenders on the Different Sperm Kinematic Parameters

As seen in *Table 4,* the results showed that the straight line velocity (VSL), curvilinear velocity (VCL), average path velocity (VAP), amplitude of lateral head displacement (ALH), and average motion degree (MAD) of sperm decreased with the extension of storage time. Among them, the decline of extender A was steady and slow. At 24 h of preservation, the VSL of sperm preserved in extender A was significantly higher than that in extenders B, C and E (Control) (P≤0.05), but not significantly different from that in extender D (P>0.05). At 24 h of preservation, the wobble movement coefficient (WOB) of sperm preserved in extender A was

Table 3. *Effects of different extenders on the progressive motility of preserved sperm (mean±SD); %*

Storage Time	A	B	C	D	E (Control)
0 h	79.58±5.17^{ab}	83.93±8.70^{a}	70.34±1.35^{b}	72.97±5.66^{b}	78.44±5.50^{ab}
24 h	79.52±5.60^{a}	51.59±3.05^{b}	5.84±3.08^{e}	33.64±2.21^{c}	0.59±0.20^{e}
48 h	72.10±1.82^{a}	45.79±3.37^{b}	0.36±0.28^{e}	8.10±2.42^{d}	-
72 h	63.01±6.07^{a}	35.54±5.62^{b}	-	8.26±1.50^{c}	-
96 h	42.83±10.48^{a}	19.25±5.50^{b}	-	5.36±2.44^{c}	-
120 h	17.07±5.65^{a}	6.40±3.75^{b}	-	0.89±0.27^{b}	-
144 h	2.04±1.18^{b}	0.83±0.86^{b}	-	0.24±0.01^{b}	-

$^{a-e}$ Values within the same row with different letters differ significantly (P≤0.05); - means that the sperm have all died, losing the significance of detection

Table 4. *Effects of different extenders on the different kinematic parameters of preserved sperm (mean±SD)*

Kinematic Parameter	Storage Time	A	B	C	D	E (Control)
VSL (µm/s)	0 h	38.01±0.^{88a}bc	39.21±2.74^{ab}	40.03±1.86^{a}	35.63±1.72^{c}	40.58±0.69^{a}
	24 h	35.91±2.68^{a}	24.09±1.21^{bc}	16.63±2.89^{c}	26.79±0.50^{ab}	14.61±11.57^{c}
	48 h	30.44±3.00^{a}	26.28±0.81^{b}	5.90±3.38^{d}	19.17±0.94^{c}	-
	72 h	27.24±1.78^{a}	24.29±1.59^{ab}	-	22.65±1.33^{bc}	-
	96 h	21.94±2.45^{a}	21.24±2.03^{a}	-	18.86±4.2^{a} 6	-
	120 h	17.58±1.49^{a}	17.42±1.75^{a}	-	11.58±3.16^{b}	-
	144 h	12.28±2.81^{a}	8.68±9.13^{a}	-	4.64±0.42^{a}	-
VCL (µm/s)	0 h	73.80±2.37^{a}	71.34±1.27^{a}	71.29±3.89^{a}	63.18±3.08^{b}	73.27±4.97^{a}
	24 h	76.01±3.98^{a}	57.55±2.68^{b}	37.20±7.86^{c}	56.23±0.38^{b}	27.86±20.64^{c}
	48 h	65.19±4.77^{a}	58.04±2.82^{b}	8.90±4.13^{e}	39.39±0.76^{d}	-
	72 h	59.10±3.31^{a}	43.67±5.89^{b}	-	41.40±2.51^{b}	-
	96 h	47.58±4.17^{a}	39.85±5.80^{a}	-	44.60±5.93^{a}	-
	120 h	40.61±3.18^{a}	31.66±2.57^{b}	-	34.03±6.78^{ab}	-
	144 h	29.65±9.64^{a}	15.60±17.07^{ab}	-	8.39±2.45^{b}	-
VAP(µm/s)	0 h	52.19±1.68^{a}	50.45±0.90^{a}	50.41±2.75^{a}	44.67±2.17^{b}	51.81±3.52^{a}
	24 h	53.75±2.81^{a}	40.69±1.89^{b}	26.31±5.56^{c}	39.76±0.27^{b}	19.70±14.59^{c}
	48 h	46.10±3.37^{a}	41.04±1.99^{b}	6.29±2.92^{e}	27.85±0.54^{d}	-
	72 h	41.79±2.34^{a}	30.88±4.17^{b}	-	29.28±1.78^{b}	-
	96 h	33.64±2.95^{a}	28.18±4.10^{a}	-	31.53±4.19^{a}	-
	120 h	28.72±2.25^{a}	22.38±1.82^{b}	-	24.06±4.79^{ab}	-
	144 h	20.97±6.81^{a}	11.03±12.07^{ab}	-	5.94±1.74^{b}	-
ALH (µm)	0 h	21.62±0.70^{a}	20.90±0.38^{a}	20.88±1.14^{a}	18.51±0.90^{b}	21.46±1.46^{a}
	24 h	22.26±1.17^{a}	16.86±0.78^{b}	10.90±2.31^{c}	16.47±0.11^{b}	8.16±6.05^{c}
	48 h	19.10±1.40^{a}	17.00±0.83^{b}	2.60±1.21^{e}	11.54±0.23^{d}	-
	72 h	17.31±0.97^{a}	12.79±1.73^{b}	-	12.13±0.74^{b}	-
	96 h	13.93±1.22^{a}	11.67±1.70^{a}	-	13.07±1.74^{a}	-
	120 h	11.89±0.93^{a}	9.27±0.76^{b}	-	9.97±1.99^{ab}	-
	144 h	8.69±2.82^{a}	4.57±5.00^{ab}	-	2.46±0.72^{b}	-
LIN (%)	0 h	0.52±0.01^{bc}	0.55±0.03^{abc}	0.57±0.01^{a}	0.56±0.03^{ab}	0.55±0.03^{abc}
	24 h	0.47±0.01^{a}	0.42±0.00^{a}	0.46±0.02^{a}	0.47±0.01^{a}	0.35±0.2^{a} 0
	48 h	0.47±0.01^{a}	0.45±0.01^{a}	0.30±0.17^{b}	0.49±0.02^{a}	-
	72 h	0.46±0.01^{b}	0.56±0.05^{a}	-	0.55±0.01^{a}	-
	96 h	0.46±0.01^{b}	0.53±0.03^{a}	-	0.43±0.04^{bc}	-
	120 h	0.43±0.02^{b}	0.55±0.01^{a}	-	0.30±0.09^{c}	-
	144 h	0.38±0.08^{a}	0.32±0.29^{a}	-	0.20±0.04^{a}	-

$^{a-e}$ Values within the same row with different letters differ significantly (P≤0.05); - means that the sperm have all died, losing the significance of detection

Table 4. *Effects of different extenders on the different kinematic parameters of preserved sperm (mean±SD) (continued...)*

Kinematic Parameter	Storage Time	A	B	C	D	E (Control)
STR (%)	0 h	0.73±0.01^{ab}	0.78±0.05^{ab}	0.80±0.01^{a}	0.80±0.04^{a}	0.79±0.04^{ab}
	24 h	0.67±0.02^{a}	0.59±0.01^{a}	0.64±0.03^{a}	0.67±0.01^{a}	0.49±0.29^{a}
	48 h	0.66±0.02^{a}	0.64±0.01^{a}	0.42±0.24^{b}	0.69±0.04^{a}	-
	72 h	0.65±0.01^{b}	0.79±0.06^{a}	-	0.77±0.00^{a}	-
	96 h	0.65±0.02^{b}	0.76±0.05^{a}	-	0.60±0.05^{bc}	-
	120 h	0.62±0.03^{b}	0.79±0.02^{a}	-	0.42±0.13^{c}	-
	144 h	0.53±0.10^{a}	0.46±0.41^{a}	-	0.28±0.06^{a}	-
BCF (Hz)	0 h	0.70±0.02^{ab}	0.72±0.02^{a}	0.65±0.02^{b}	0.67±0.02^{b}	0.69±0.04^{ab}
	24 h	0.72±0.03^{a}	0.69±0.02^{a}	1.20±0.73^{a}	0.66±0.01^{a}	0.78±0.51^{a}
	48 h	0.70±0.02^{b}	0.67±0.02^{b}	1.94±1.20^{a}	0.78±0.02^{b}	-
	72 h	0.69±0.02^{b}	0.68±0.06^{b}	-	0.82±0.07^{a}	-
	96 h	0.74±0.05^{a}	0.78±0.05^{a}	-	0.78±0.14^{a}	-
	120 h	0.77±0.06^{a}	1.89±1.65^{a}	-	1.20±0.70^{a}	-
	144 h	3.16±1.72^{a}	1.69±1.47^{a}	-	1.17±0.50^{a}	-
MAD (°/s)	0 h	152.87±27.42^{ab}	195.79±61.33^{a}	119.43±12.58^{b}	146.90±33.40^{ab}	122.90^{b}±30.90
	24 h	183.35±73.24^{a}	128.03±6.13^{b}	13.36±5.55^{cd}	66.77±11.49^{c}	1.94^{d}±0.91
	48 h	153.32±29.32^{a}	102.66±19.58^{b}	3.20±1.30^{c}	19.17±6.20^{c}	-
	72 h	107.91±4.90^{a}	63.74±13.19^{b}	-	18.63±2.49^{c}	-
	96 h	92.37±19.59^{a}	39.20±11.28^{b}	-	13.14±2.11^{c}	-
	120 h	39.98±6.80^{a}	12.41±5.04^{b}	-	3.99±1.78^{b}	-
	144 h	16.47±10.28^{a}	3.46±3.03^{b}	-	1.89±0.46^{b}	-
WOB (%)	0 h	0.87±0.01^{ab}	0.83±0.05^{bc}	0.80±0.02^{bc}	0.79±0.03^{c}	0.83±0.05^{bc}
	24 h	0.94±0.02^{a}	0.92±0.03^{a}	0.86±0.04^{a}	0.91±0.01^{a}	0.61±0.26^{b}
	48 h	0.94±0.02^{a}	0.92±0.03^{a}	0.17±0.17^{b}	0.88±0.02^{a}	-
	72 h	0.92±0.02^{a}	0.76±0.09^{b}	-	0.89±0.03^{a}	-
	96 h	0.94±0.02^{a}	0.79±0.07^{b}	-	0.94±0.07^{a}	-
	120 h	0.95±0.04^{a}	0.84±0.06^{a}	-	0.84±0.17^{a}	-
	144 h	0.85±0.17^{a}	0.53±0.48^{ab}	-	0.17±0.17^{b}	-

$^{a-e}$ Values within the same row with different letters differ significantly (P≤0.05); - means that the sperm have all died, losing the significance of detection

the highest, and there was a significant difference between extender A and extender E (Control) (P≤0.05). At 48 h of preservation, the VSL of sperm preserved in extender A was significantly higher than those in the other four extenders (P≤0.05). The WOB of sperm preserved in extender A was the highest, and there was significant difference between extender A and extender C (P≤0.05). At 72 and 96 h, the WOB of sperm preserved in extender A was the highest and significantly higher than that in extender B (P≤0.05), but there was no significant difference between extender A and extender D (P>0.05). The VSL of sperm preserved in extender A was the highest at 72 h and 120 h, and there was a significant difference between extender A and extender D (P≤0.05), but there was no significant difference between extender A and extender B (P>0.05). At 96 and 144 h, the VSL of sperm preserved in extender A was the highest, but there was no significant difference between extender A and the other extenders (P>0.05). The VCL, VAP and ALH of sperm preserved in extender A were significantly higher than those in the other four extenders within 24 to 72 h (P≤0.05). At 96 h of preservation, the VCL, VAP and ALH of sperm preserved in extender A were the highest, but there were no significant differences between extender A and the other extenders (P>0.05). At 120 h of preservation, the VCL, VAP and ALH of sperm preserved in extender A were the highest and were significantly higher than those in extender B (P≤.05), but there was no significant difference between extender A and extender D (P>0.05). At 120 h of preservation, the WOB of sperm preserved in extender A was the highest, but there was no significant difference compared with the other extenders (P>0.05). At 144 h of preservation, the VCL, VAP, ALH and WOB of sperm preserved in extender A were the highest, and there were significant differences between extender A and extender D, but there was no significant difference between extender A and extender B (P>0.05). At 144 h of preservation, the LIN, STR and BCF of sperm preserved in extender A were the highest, but there was no significant difference between extender A and the other extenders (P>0.05). The sperm MAD of extender A was significantly

Table 5. *Effects of different extenders on the survival time of preserved sperm (mean±SD); h*					
Survival Time	**A**	**B**	**C**	**D**	**E (Control)**
Effective survival time	74.50±4.82[a]	21.83±0.76[b]	2.00±0.50[d]	12.83±1.04[c]	5.83±1.04[d]
Total survival time	412.67±2.52[a]	219.83±6.21[b]	63.67±1.53[d]	186.67±7.64[c]	35.50±1.32[e]
[a-e] Values within the same row with different letters differ significantly (P≤0.05)					

Table 6. *Effects of different extenders on the integrity of plasma membrane of preserved sperm (mean±SD); %*				
Storage Time	**A**	**B**	**D**	**E (Control)**
0 h	61.82±1.61[b]	62.44±1.24[b]	63.12±1.69[ab]	65.65±1.02[a]
24 h	56.38±4.99[a]	54.80±3.97[a]	40.18±2.79[b]	21.13±3.76[c]
48 h	55.45±3.99[a]	51.25±2.81[a]	35.84±5.69[b]	-
72 h	47.38±1.23[a]	46.88±0.54[a]	35.61±0.86[b]	-
96 h	45.35±1.58[a]	42.23±2.41[a]	32.23±1.94[b]	-
120 h	40.75±2.12[a]	42.21±0.52[a]	32.18±1.01[b]	-
144 h	34.00±4.00[a]	37.67±3.40[a]	26.89±0.44[b]	-
[a-c] Values within the same row with different letters differ significantly (P≤0.05); - means that the sperm have all died, losing the significance of detection. The effective survival time of sperm preserved by extender C was lower than that of the normal saline group, so the detection of plasma membrane integrity was meaningless				

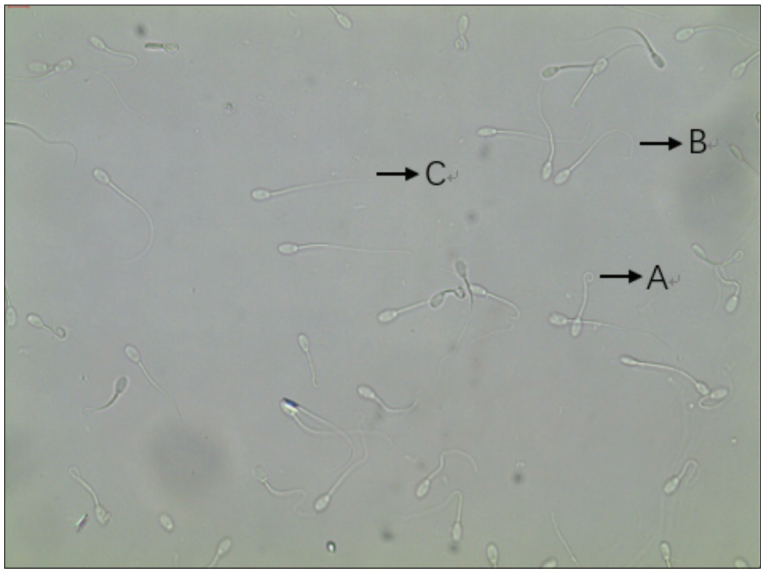

Fig 1. Morphology of curly tail of sperm in HOST. Observation under 400x lens, the two types of tail curl A and B were intact plasma membrane sperm, and the tail non-curl type C was the sperm with damaged plasma membrane

higher than those of the other four extenders within 24 to 144 h (P≤0.05).

Effects of Different Extenders on the Sperm Survival Time

As seen in *Table 5,* the effective and total survival times of semen preserved in extender A were the highest and were significantly higher than those of the other four extenders (P≤0.05).

Effects of Different Extenders on the Sperm Plasma Membrane Integrity

The effective survival time of sperm preserved by extender C was lower than that of the normal saline group; therefore, the detection of plasma membrane integrity was meaningless.

Table 6 shows that when Hu ram semen was preserved with four kinds of extenders at 16°C, the integrity of sperm plasma membrane decreased with the extension of storage time. Among them, the plasma membrane integrity of sperm preserved by extender A decreased steadily and slowly. At 24 h of preservation, the integrity of the plasma membrane of sperm preserved in extender A was the highest and was significantly higher than those in extenders D and E (Control) (P≤0.05), but there was no significant difference between extender A and extender B (P>0.05). Within 48 to 144 h of preservation, the integrity of the plasma membrane of sperm preserved in extender A was higher than those in the other three extenders.

The results of microscopic examination after HOST incubation are shown in *Fig. 1*. There were three types of sperm tail:

Table 7. *Effects of different extenders on acrosome integrity of preserved sperm (mean±SD); %*

Storage Time	A	B	D	E (Control)
0 h	96.45±1.7[a]a	94.62±2.67[ab]	93.44±1.54[ab]	92.53±2.37[b]
24 h	93.66±1.16[a]	89.00±1.81[ab]	83.47±3.40[bc]	79.44±6.55[c]
48 h	93.00±1.43[a]	88.08±2.79[b]	76.43±1.28[c]	-
72 h	92.55±1.23[a]	87.21±1.13[b]	70.01±4.05[c]	-
96 h	92.50±0.79[a]	85.48±0.71[b]	66.69±3.82[c]	-
120 h	92.21±0.73[a]	85.04±2.15[b]	64.47±3.70[c]	-
144 h	90.93±1.88[a]	84.43±1.07[b]	49.45±1.24[c]	-

[a-c] Values within the same row with different letters differ significantly (P≤0.05); - means that the sperm have all died, losing the significance of detection. The effective survival time of sperm preserved by extender C was lower than that of the normal saline group, so the detection of acrosome integrity was meaningless

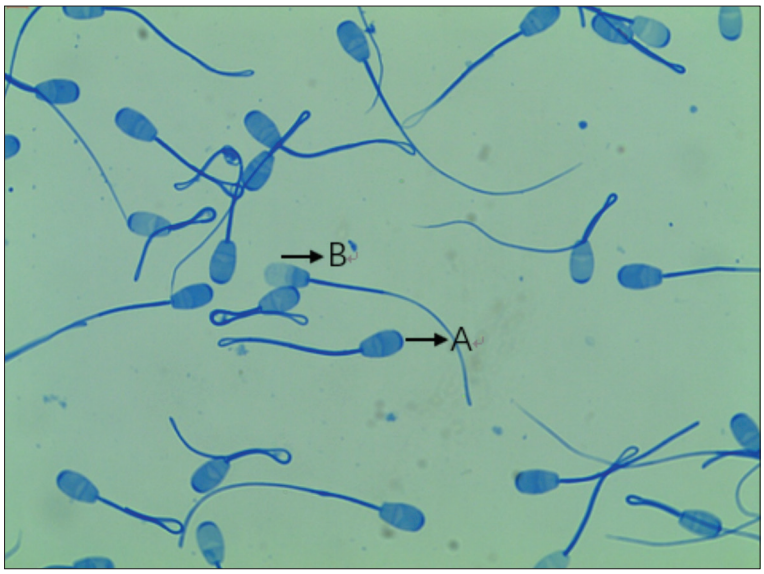

Fig 2. Acrosome morphology of sperm stained with Coomassie brilliant blue. Observation under 1000x oil lens, there were two types of sperm head: type A and type B. Sperm head is blue, it means the acrosome is intact(A). Sperm head was unstained, it means the acrosome is not intact (B)

A, B and C, in which the two types of tail curl, A and B, represented intact plasma membrane sperm, and the tail non-curl type C represented sperm with damaged plasma membrane.

Effects of Different Extenders on Sperm Acrosome Integrity

The effective survival time of sperm preserved by extender C was lower than in the normal saline group; therefore, the detection of acrosome integrity was meaningless.

Table 7 shows that when Hu ram semen was preserved with four kinds of extenders at 16°C, the acrosome integrity of sperm decreased with the extension of storage time. Among them, the acrosome integrity of sperm preserved in extender A decreased steadily and slowly. At 24 h of preservation, the acrosome integrity of sperm preserved in extender A was the highest and was significantly higher than those in extender D and extender E (Control) (P≤0.05), but there was no significant difference between extender A and extender B (P>0.05). Within 48 to 96 h of preservation, the acrosome integrity of sperm preserved in extender A was higher than those of the other three extenders.

The results of microscopic examination after Coomassie brilliant blue staining are shown in *Fig. 2*. There were two types of sperm head: type A and type B. If the sperm head is blue, then the acrosome is intact (A). If the sperm head was unstained, then the acrosome is not intact (B).

Discussion

In this study, total sperm motility, sperm progressive motility, effective survival time, total survival time, plasma membrane integrity, acrosome integrity and some kinematic parameters such as VSL, VCL and VAP were used to analyze the effects of different kinds of extenders on the preservation of Hu ram semen at 16°C. The results showed that the total sperm motility and progressive motility of extender A decreased steadily and slowly within 144 h of preservation of Hu ram semen at 16°C, and the performance of extender A was obviously better than those of the other extenders. The effective survival time, total survival time, plasma membrane integrity, acrosome integrity and motility performance of semen preserved in extender A were also significantly better than those of the other extenders. In short, extender A

has the best effect on the preservation of Hu ram semen at 16°C.

At present, the extender preservation of ram semen has been studied in many aspects [18]. At present, most of the studies on ram semen are 4°C preservation and cryopreservation, but there are a few reports on 16°C preservation. No matter which preservation method is used, the preservation quality of ram semen decreases gradually with time [19]. It has been reported that the storage time of ram semen at 16°C is too short. Yaniz et al.[20] indicated that the sperm progressive motility was less than 60% at 24 h in the ram semen preservation experiment at 16°C, which was far from meeting the production needs [21]. Compared with 16°C preservation, the extender formula and operation process of semen 4°C preservation are more complex, which is not conducive to popularization and application, and 4°C preservation may have a certain impact on the sperm membrane [22]. At the same time, 4°C preservation may also lead to the decrease of fertilization ability and an increased embryo loss rate [23,24].

At present, commercial extenders are used in the preservation of ram semen at 16°C, and there are few published reports on extender formulations. In the study of 4°C preservation of ram semen, Sarlos et al.[25] used an extender composed of Tris, citric acid, glucose, yolk and antibacterial substances, and Kasimanickam et al.[26] and Gundogan et al.[27] used an extender composed of Tris, citric acid, fructose, yolk and antibacterial substances. In the study of cryopreservation of ram semen, Kumar et al.[28] and Leahy et al.[29] used a basic extender composed of Tris, citric acid, fructose, yolk and antibacterial substances, and Hamedani et al.[30] and Merati et al.[31] used extenders composed of Tris, citric acid, glucose, yolk, glycerol and antibacterial substances. In this experiment, various substances of extender A formula are used in Hu ram semen 4°C preservation and cryopreservation, and nutrients in previous formulations, such as glucose and fructose, are also used. Yolk and glycerol are added in the 4°C preservation and cryopreservation of ram semen. Yolk and glycerol are used as effective cryoprotectants. Additionally, yolk could also supply energy to sperm. Therefore, in experiments evaluating the preservation of Hu ram semen at 16°C, if yolk is not added, the amount of nutrients such as glucose or fructose should be increased.

The extender is composed of nutrients, buffers, antibiotics and other substances. The pH, osmotic pressure, buffering properties and functional substances of the extender will affect the preservation quality of semen [32]. In this experiment, the sudden decreases of total motility and progressive motility of Hu ram sperm stored in extenders C and D at 16°C may have been due to the lack of substances to regulate and stabilize the pH in the extenders. In this experiment, the extender E (Control) was a 0.9% sodium chloride extender, and the sudden decline of total sperm motility during preservation may be due to the lack of nutrients in the extender, which cannot provide energy for sperm metabolism. In this experiment, the effective survival time of Hu ram sperm preserved in extender C at 16°C was lower than that in extender E (Control), normal saline, which may be due to the changes of the physical and chemical properties of the extender, which was not suitable for sperm survival. The total survival time of Hu ram sperm preserved in extender E (Control) at 16°C was lower than those with extender C and extender D, which may be because it did not contain nutrients and could not provide energy for sperm metabolism. The acrosome of sperm is an important organelle in the process of fertilization, and the survival time is proportional to the acrosome integrity [33]. The acrosome integrity obtained in this experiment is proportional to the survival time, which is consistent with previous research results. VSL, VCL, VAP, WOB, ALH and BCF are single variables describing sperm velocity and are closely related to reproductive performance [34]. VSL, VCL and VAP are the key parameters to evaluate sperm kinematic parameters, and the fertilization ability is coordinately proportional [35]. Deyiliu showed that there is a significant relationship between ALH, BCF and semen quality [36]. The VSL, VCL, VAP, ALH and WOB obtained in this experiment are proportional to sperm progressive motility, which is consistent with the results of previous studies. The MAD obtained in this experiment is proportional to sperm progressive motility, and there is no correlation between other kinematic parameters and sperm progressive motility.

In conclusion, the experimental results show that extender A is the best formula among the five extenders. At present, there are few extenders for Hu ram semen stored at 16°C. The storage time of the existing 16°C storage extender is too short. Although Tris extender is often used for cryopreservation, it has not been reported under 16°C storage conditions. In this experiment, the cryoprotective agent in the Tris extender during cryopreservation was removed. The formulation was optimized and was applied to 16°C storage, which greatly extended the survival time of sperm. The preparation process of extender is simple and easy to operate, which can meet the practical requirements of production and has good popularizing value.

Statement of Author Contributions

LZ and YL: conceptualization. LZ, TS and CC: methodology. LZ and YW: software. YF: validation. LZ, YW and CC: formal analysis. LZ and JM: investigation. YL: resources. YW and YF: data curation. YL: supervision and validation. LZ: writing - original draft preparation. YL: writing - review and editing. All authors discussed the results and contributed to the final manuscript.

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Conflict of Interest

None of the authors have any conflict of interest to declare.

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