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RESEARCH ARTICLE

The Effect of Different Storage Temperature on Hu Ram Sperm Parameters

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Abstract: In order to explore the most suitable temperature for the preservation of Hu ram semen at room temperature, the semen samples were placed at 15°C, 20°C, and 25°C respectively. The energy metabolism of enzyme activity and triphosphate adenosine (ATP) was detected in the process of preservation. Different parameters such as functional integrity, survival time, reactive oxygen species (ROS) content, superoxide dismutase (SOD) activity, and energy metabolism of adenosine triphosphate (ATP) were detected. The results showed that within 2 days of storage, the sperm progressive motility stored at 25°C was the highest. Within 3~5 days of storage, sperm stored at 20°C have the highest progressive motility. Within 6~9 days of storage, the sperm progressive motility stored at 15°C was significantly higher than that of the other groups (P<0.05). The effective survival time and total survival time of sperm stored at 15°C reached 5.67 d and 18.73 d, respectively, which were significantly higher than those of other groups (P<0.05). On the 1st day of storage, the sperm membrane integrity was significantly higher than that of the other groups (P<0.05). Within 3~5 days of storage, the membrane integrity and acrosome integrity of sperm stored at 15°C and 20°C were significantly higher than the sperm membrane integrity and acrosome integrity stored at 25°C (P<0.05). On the 7th day of storage, the membrane integrity of sperm stored at 15°C was significantly higher than that of other groups (P<0.05). On the 5th day of storage, the level of sperm ROS stored at 15°C was significantly lower than that of group 25°C (P<0.05), SOD activity, Catalase enzyme (CAT) activity and ATP content were significantly higher than that of the other groups (P<0.05). Therefore, compared with 20°C and 25°C, 15°C was the most suitable temperature for the preservation of Hu ram semen at room temperature.

Keywords: *Temperature, Sperm, Motility parameters, Physiological characteristics, Oxidative stress*

Farklı Saklama Sıcaklıklarının Hu Koç Sperm Parametreleri Üzerine Etkisi

Öz: Hu koç sperminin oda sıcaklığında muhafazasında en uygun sıcaklığı belirlemek için, sperm örnekleri sırasıyla 15°C, 20°C ve 25°C'de bekletildi. Muhafaza sürecinde enzim aktivitesinin enerji metabolizması ve adenosin trifosfat (ATP) ölçüldü. Fonksiyonel bütünlük, yaşama süresi, reaktif oksijen türlerinin (ROS) içeriği, süperoksit dismutaz (SOD) aktivitesi ve adenozin trifosfatın (ATP) enerji metabolizması gibi farklı parametreler ölçüldü. Bulgular, 2 günlük muhafaza süresince 25°C'de bekletilen spermlerin progresif hareketliliğinin en yüksek olduğunu gösterdi. 3~5 günlük muhafaza süresince, 20°C'de bekletilen spermler en yüksek progresif hareketliliğe sahipti. 6~9 günlük depolama süresince, 15°C'de bekletilen spermlerin progresif motilitesi diğer gruplara göre önemli ölçüde daha yüksekti (P<0.05). 15°C'de muhafaza edilen spermlerin etkin canlı kalma süresi ve toplam canlı kalma süresi, diğer gruplardan önemli ölçüde daha yüksekti ve sırasıyla 5.67 gün ve 18.73 güne ulaştı (P<0.05). Muhafazanın 1. gününde, sperm membran bütünlüğü diğer gruplara göre anlamlı derecede yüksekti (P<0.05). Muhafazadan sonraki 3~5 gün içinde, 15°C ve 20°C'de saklanan spermin membran bütünlüğü ve akrozom bütünlüğü, 25°C'de saklanan spermin membran bütünlüğü ve akrozom bütünlüğünden önemli ölçüde daha yüksekti (P<0.05). Muhafazanın 7. gününde 15°C'de saklanan spermin membran bütünlüğü diğer gruplara göre anlamlı derecede yüksekti (P<0.05). Muhafazanın 5. gününde, 15°C'de saklanan spermin ROS seviyesi, 25°C'de saklanan grubtan anlamlı derecede (P<0.05) düşük iken, SOD aktivitesi, katalaz enzim (CAT) aktivitesi ve ATP içeriği diğer gruplardan önemli ölçüde daha yüksekti (P<0.05). Dolayısıyla, 20°C ve 25°C ile karşılaştırıldığında, Hu koç sperminin korunması için en uygun oda sıcaklığı 15°C olarak saptandı.

Anahtar sözcükler: *Sıcaklık, Sperm, Motilite parametreleri, Fizyolojik özellikler, Oksidatif stres*

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Introduction

Hu sheep is a world-famous high fecundity sheep breed and a first level protected local livestock breed in China [1,2]. Hu sheep is known for their high fertility and the breeding scale is gradually expanding [3-5].

In order to improve the reproduction efficiency of Hu sheep, to make full use of the semen of excellent breeding males, to reduce breeding costs, and to avoid the spread of diseases caused by natural mating; Artificial Insemination (AI) has been applied to the breeding of Hu sheep in actual production [6,7]. Semen preservation is the core part of AI; which is mainly divided into room temperature preservation, 4°C and cryopreservation [8]. Because sheep sperm membranes contain a large amount of unsaturated fatty acids, they are susceptible to irreversible low temperature damage, destroying the complete structure of sperm, causing sperm to lose physiological functions and internal material loss, thereby affecting the quality of semen preservation [9]. On the other hand, cryopreservation not only requires expensive instruments, but also high technical requirements, which limits its application in production practice. Cryopreservation causes a certain cold shock to sperm, and there are also certain technical requirements. Therefore, in actual production, room temperature preservation is widely used, which is more conducive to improve the utilization rate of genetic resources of breeding rams [10]. Optimum temperature is essential for semen preservation. When the storage temperature of semen increases, the metabolism and respiration of sperms enhance, and the movement and energy consumption increase. When the temperature rises to a certain level, the decomposition of the sperm itself will increase the ammonia content and increase the pH of the semen, which will further enhance the metabolism and respiration of the sperm [11]. When the temperature rises, the reproduction speed of bacteria in semen will also increase, producing a large amount of metabolic waste, damaging sperm cells, and affecting the preservation quality of semen [12]. When the temperature drops to a certain level, it will affect the physiological function of the sperm or destroy the sperm structure [13]. For example, the optimal temperature for storage of pig semen at room temperature is 17°C. 15°C~25°C belong to the range of room temperature preservation, but there is no uniform standard for the optimum temperature for storage of Hu ram semen at room temperature.

In this experiment, different storage temperatures (15°C, 20°C, and 25°C) were assumed to store Hu ram semen at room temperature. Aim was to detect and analyze of Hu ram sperm motility parameters such as total motility, progressive motility, survival time and membrane integrity. Other functional integrity parameters such as ROS level, SOD activity oxidative stress parameters and ATP content under different room temperature storage of semen. The basic object of the study was to obtain optimal temperature for storage of Hu ram semen at room temperature.

Material and Methods

Ethics Statement

All animal procedures confirm to the guidelines and regulatory standards of the Animal care committee of the Yangzhou University (Approval ID: SYXK [Su] 2017-0044).

Experimental Design

Five experiments were conducted to evaluate the effects of different temperatures on semen preservation of Hu rams at room temperature.

Experiment 1: The effect of different temperatures (15°C, 20°C, and 25°C) to the Hu ram semen preserved at room temperature on the motility parameters of sperm. Sperm total motility, progressive motility, and kinematic parameters of the three groups were evaluated.

Experiment 2: The effect of different temperatures (15°C, 20°C, and 25°C) to the Hu ram semen preserved at room temperature on the survival time of sperm. Effective survival time, and total survival time of the three groups were evaluated.

Experiment 3: The effect of different temperatures (15°C, 20°C, and 25°C) to the Hu ram semen preserved at room temperature on the physiological characteristics of sperm. Membrane integrity, and Acrosome integrity of the three groups were evaluated.

Experiment 4: The effect of different temperatures (15°C, 20°C, and 25°C) to the Hu ram semen preserved at room temperature on the antioxidant parameters of sperm. ROS, Malondialdehyde (MDA), SOD, and CAT of the three groups were evaluated.

Experiment 5: The effect of different temperatures (15°C, 20°C, and 25°C) to the Hu ram semen preserved at room temperature on the ATP of sperm.

Preparation of Semen Extender and Semen Collection

The extender consisted of 15.35 g Tris, 10.00 g fructose, 8.20 g citric acid and 250.000 IU each of penicillin and streptomycin in 500 mL distilled water.

Semen samples were collected from three Hu rams aged two years with the aid of an artificial vagina. A total number of 50 ejaculates were collected from three rams. The collected semen volume of each ram was about 1.0 mL every time. Sperm concentration reached $2.3x10^9$/mL. The three rams were raised in the experimental sheep farm of Yangzhou University. The rams were fed 0.2 kg concentrate twice a day, and *ad libitum* hay and water. The experimental farm is equipped with licking bricks

for sheep to lick freely, so as to ensure sufficient minerals and microelements. There is also an open area in the experimental farm to ensure that the sheep have enough exercise. Semen samples were evaluated for sperm motility using computer-assisted sperm analyzer (CASA). Ejaculates showing >75% motility were pooled to minimize individual differences.

Dilution and Evaluation of Semen

The pooled fresh semen samples were split into three equal fractions in different test tubes and diluted at room temperature with a Tris extender. The processed semen was stored respectively in a 15°C, 20°C, and 25°C incubator and the semen was gently flipped every day.

Evaluation of Sperm Motility

Semen samples were evaluated for sperm kinetics using CASA (ML-608JZ II Mailang, Nanning, China).

Evaluation of Sperm Survival Time

The time when the sperm progressive motility is above 60% is called the effective survival time. The time when all sperm died is called the total survival time.

Evaluation of Sperm Physiological Characteristics

The hypo-osmotic swelling test (HOST) was used to evaluate the sperm membrane integrity. 10 μL of preserved semen and 100 μL of hypo-osmotic solution were mixed and incubated at 37°C for at least 30 min. Five μL of suspension was loaded on a slide and 200 cells with swollen and non-swollen tails were counted as sperm with membrane integrity and non-integrity respectively, under a 400x phase-contrast microscope. Acrosome integrity was detected by Coomassie brilliant blue staining. Fifty μL of preserved semen and 1 mL of 4% paraformaldehyde were mixed and fixed at room temperature for 10 min. After centrifugation at 1500x g for 5 min, the supernatant was discarded, and 10 μL of semen taken to make a smear. After air-drying, this was stained with Coomassie Brilliant Blue dye for at least 30 min, rinsed with water, and air-dried. Then 200 cells with head stained blue and unstained blue were counted as sperm with acrosome integrity and non-integrity under a 1000x oil immersion.

Evaluation of Sperm Antioxidant Parameters and ATP Content

ROS level of sperm was measured using a ROS Assay Kit (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's instruction. The ROS level was expressed by the fluorescence intensity. Fluorescence intensity of DCF (488 nm excitation and 525 nm emission for DCF) was detected by a multifunctional microplate reader.

The MDA content in the semen was measured using a Lipid Peroxidation MDA Assay Kit (Beyotime Institute of Biotechnology) according to the manufacturer's instruction. Absorbance at 532 nm was detected by a multifunctional microplate reader. Finally, the results were obtained according to the standard curve.

SOD activity of sperm was measured using a Total Superoxide Dismutase Assay Kit with WST-8 (Beyotime Institute of Biotechnology) according to the manufacturer's instruction. Add the reagents in order according to the procedure. Absorbance at 450 nm was detected by a multifunctional microplate reader. Finally, we got the result according to the formula in the instruction.

CAT activity in the semen was measured using a CAT Assay Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instruction. Add the reagents in order according to the procedure. Finally, we got the result according to the formula in the instruction.

The ATP content in the semen was measured using ATP Assay Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instruction. Add the reagents in order according to the procedure. Absorbance at 636 nm was detected by a multifunctional microplate reader. Finally, we got the result according to the formula in the instruction.

Statistical Analysis

Data were analyzed using SPSS 25.0 software. Significance was set at $P<0.05$ unless otherwise specified. The results were expressed as the Mean ± SEM. One-way ANOVA tests were performed to assess the difference in these parameters.

Results

Effects of Different Temperature on Sperm Motility

The effects of different temperature on Hu ram sperm motility during liquid storage were shown in *Fig. 1*. Sperm total and progressive motility decreased with the increasing storage time *in vitro*. After 6 days preservation, the total motility and progressive motility of sperm storage at 15°C were higher than those of other groups ($P<0.05$).

Effects of Different Temperature on Sperm Kinematic Parameters

The effects of different temperature on Hu ram sperm kinematic parameters during liquid storage were shown in *Table 1*. Within 6~9 days, the Straight line velocity (VSL), Curvilinear velocity (VCL), Average path velocity (VAP), Amplitude of lateral head displacement (ALH) and Average motion degree (MAD) in the 15°C group were higher ($P<0.05$) than those in other groups.

Effects of Different Temperature on Sperm Survival Time

As shown in *Fig. 2*, the 15°C group showed the highest effective survival time and total survival time among all

Table 1. *Effects of different temperature on sperm kinematic parameters of Hu ram*

Testing Index	Storage Time (days)	Temperature		
		15°C	20°C	25°C
VSL (μm/s)	0	31.23±0.28	31.61±0.42	31.35±0.35
	1	31.70±0.67	30.92±0.81	29.43±0.87
	2	30.76±0.98	28.63±1.00	28.30±0.40
	3	27.88±0.23^{b}	29.85±0.60^{a}	30.89±0.37^{a}
	4	26.41±0.39^{b}	28.58±0.41^{a}	26.52±0.62^{b}
	5	26.67±0.22^{a}	26.67±0.42^{a}	19.17±0.08^{b}
	6	25.32±0.71^{a}	21.37±0.22^{b}	2.78±2.78^{c}
	7	26.14±0.05^{a}	19.01±0.82^{b}	2.72±1.40^{c}
	8	22.73±0.65^{a}	17.28±1.05^{b}	0^{c}
	9	25.41±0.47^{a}	11.60±0.86^{b}	0^{c}
VCL (μm/s)	0	61.06±0.27	59.93±0.36	60.44±0.37
	1	58.75±0.96	59.15±1.24	57.72±1.42
	2	58.76±1.52	55.02±3.26	55.64±0.90
	3	56.71±1.51^{b}	62.19±0.89^{a}	65.63±1.23^{a}
	4	56.27±1.78	60.46±0.94	59.68±1.42
	5	56.74±0.49^{a}	58.19±0.77^{a}	44.84±0.53^{b}
	6	59.77±2.54^{a}	51.37±0.05^{b}	4.73±4.73^{c}
	7	57.52±0.55^{a}	42.32±1.65^{b}	3.55±1.81^{c}
	8	51.58±1.62^{a}	39.07±2.44^{b}	0^{c}
	9	55.90±0.01^{a}	26.10±1.61^{b}	0^{c}
VAP (μm/s)	0	44.04±1.05	45.33±0.66	46.37±1.21
	1	41.54±0.68	41.83±0.88	40.81±1.01
	2	41.55±1.07	38.9±2.30	39.35±0.64
	3	40.10±1.07^{b}	43.97±0.63^{a}	46.41±0.87^{a}
	4	39.79±1.26	42.75±0.67	42.20±1.00
	5	40.12±0.35^{a}	41.15±0.55^{a}	31.71±0.38^{b}
	6	42.26±1.79^{a}	36.32±0.04^{b}	3.34±3.34^{c}
	7	40.67±0.39^{a}	29.92±1.17^{b}	2.51±1.28^{c}
	8	36.47±1.14^{a}	27.64±1.72^{b}	0^{c}
	9	39.53±0.01^{a}	18.4±1.145^{b}	0^{c}
ALH (μm)	0	18.24±0.43	18.78±0.27	19.21±0.50
	1	17.21±0.28	17.32±0.36	16.90±0.42
	2	17.21±0.44	16.12±0.96	16.30±0.26
	3	16.61±0.44^{b}	18.22±0.26^{a}	19.22±0.36^{a}
	4	16.48±0.52	17.71±0.28	17.48±0.42
	5	16.62±0.14^{a}	17.04±0.23^{a}	13.13±0.16^{b}
	6	17.51±0.74^{a}	15.05±0.01^{b}	1.38±1.38^{c}
	7	16.85±0.16^{a}	12.39±0.48^{b}	1.04±0.53^{c}
	8	15.11±0.48^{a}	11.45±0.71^{b}	0^{c}
	9	16.38^{a}	7.65±0.47^{b}	0^{c}
MAD (°/s)	0	146.23±14.22	149.79±5.73	145.25±6.33
	1	97.56±13.87	96.64±1.69	112.7±11.17
	2	93.59±4.27	102.46±6.17	92.78±2.35
	3	74.15±1.07	79.58±2.48	80.70±3.40
	4	69.08±6.08	97.27±13.94	73.50±8.03
	5	62.47±1.68^{a}	67.63±4.92^{a}	38.8±1.656^{b}
	6	36.38±2.64^{a}	23.50±3.99^{b}	6.3±3.295^{c}
	7	31.97±2.49^{a}	21.0±0.605^{b}	3.97±3.97^{c}
	8	28.92±1.28^{a}	17.56±2.47^{b}	0^{c}
	9	19.63±1.88^{a}	11.14±3.08^{b}	0^{c}

Data are expressed as the Mean ± SEM. Letter difference (a-c) means significant difference (P<0.05)

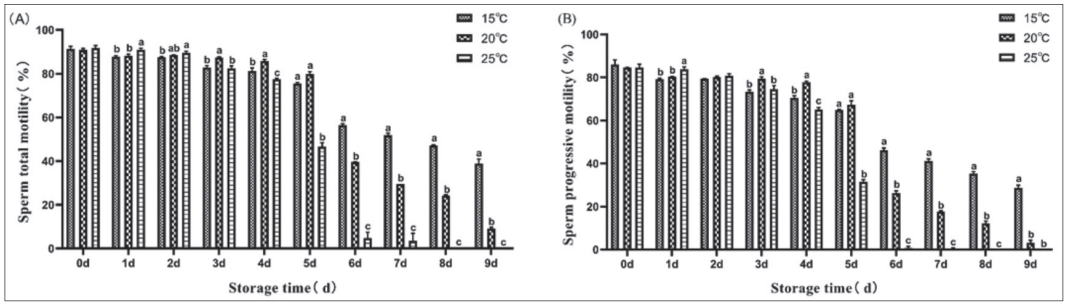

Fig 1. Effects of different temperature on sperm motility of Hu ram. (A) The percentage of sperm total motility in the 15°C, 20°C and 25°C groups; (B) The percentage of sperm progressive motility in the 15°C, 20°C and 25°C groups. The bars represent the standard error. Letter difference (a-c) means significant difference (P<0.05)

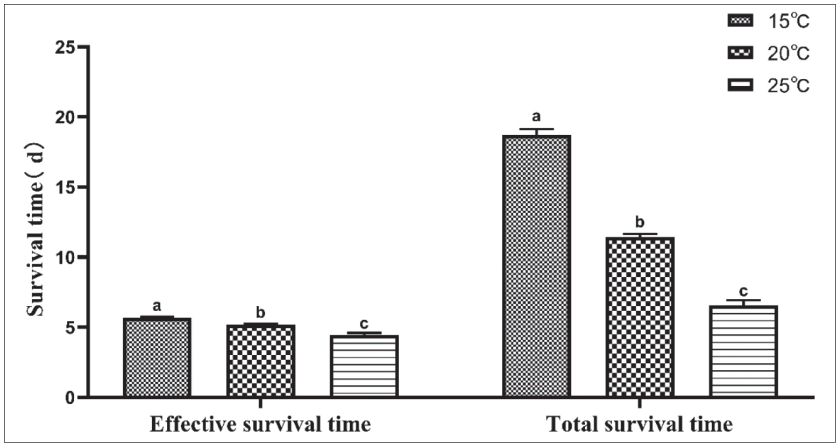

Fig 2. Effects of different temperature on sperm survival time of Hu ram. The time when the sperm progressive motility is above 60% is called the effective survival time; when the sperm total motility drops to 0, the time when all sperm die is called the total survival time. The bars represent the standard error. Letter difference (a-c) means significant difference (P<0.05)

groups (P<0.05). The effective survival time and total survival time of sperm were 5 days and 18 days, respectively.

Effects of Different Temperature on Sperm Physiological Characteristics

Microscopic examination after HOST incubation was shown in *Fig. 3-C*. There were three types of sperm tail: C, D, and E, in which the two types of tail, C and D which are curled represented intact membrane sperm, and the tail type E that is non curled represented sperm with damaged membrane. The 15°C group had the highest membrane integrity on the 7th day (P<0.05).

Microscopic examination after Coomassie brilliant blue staining was shown in *Fig. 3-D*. There were two types of sperm head: A and B. If the sperm head was blue, then the acrosome was intact (A). If the sperm head was unstained, then the acrosome was not intact (B). As shown in *Fig. 3-B*, the 25°C group had the lowest acrosome integrity within 5~7 days (P<0.05).

Effects of Different Temperature on Sperm Antioxidant Parameters

The effects of different temperature on Hu ram sperm ROS level during liquid storage were shown in *Fig. 4-A*. The sperm ROS level of the 15°C group was lower (P<0.05) than that of 25°C group on the 5th day. As shown in *Fig. 4-B*, on the 5th day, the content of MDA in 15°C group was higher than that in other groups (P<0.05). It can be seen in *Fig. 4-C* that 15°C group had the highest SOD activity on the 5th day (P<0.05). As shown in *Fig. 4-D*, on the 5th day, CAT activity of the 15°C group was higher (P<0.05) than that of 20°C group.

Effects of Different Temperature on Sperm ATP Content

The effects of different temperature on Hu ram sperm ATP content during liquid storage on the 5th day were shown in *Fig. 5*. ATP content of the 15°C group was significantly higher (P<0.05) than that of other groups.

Discussion

This study analyzed the effects of three different storage temperatures *in vitro* on the motility parameters of sperms such as sperm total motility, survival time, functional integrity such as membrane integrity, oxidative stress parameters such as ROS level, enzyme activities such as SOD activity and ATP content. Within 2 days of storage,

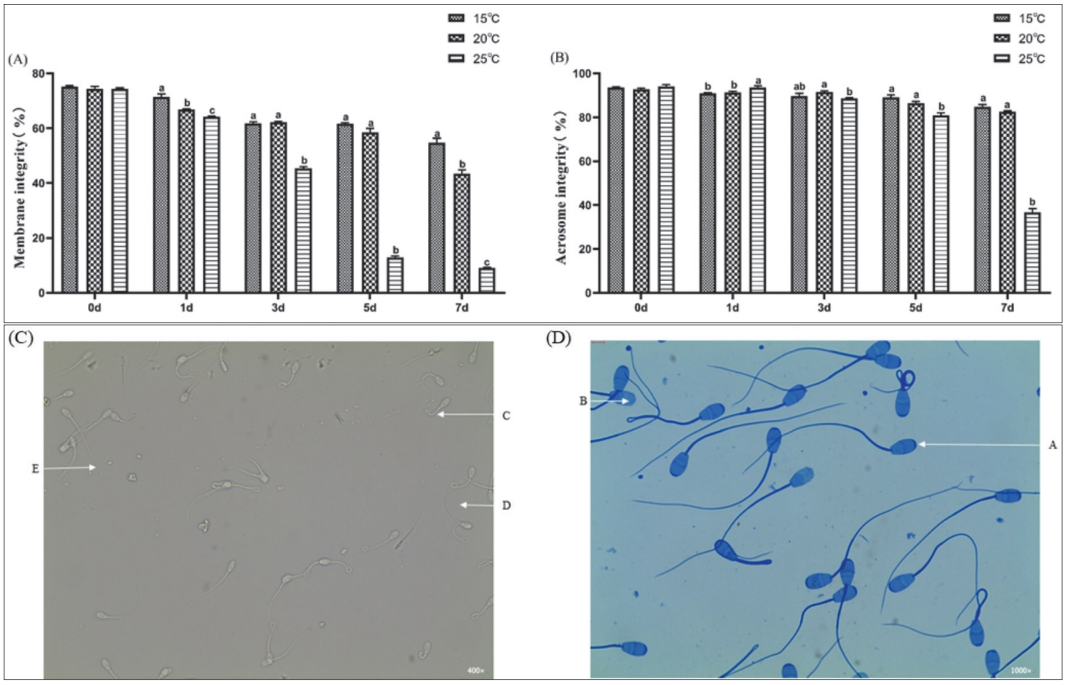

Fig 3. Effects of different temperature on sperm physiological characteristics. (A) The percentage of sperm membrane integrity in the 15°C, 20°C, and 25°C groups; (B) The percentage of sperm acrosome integrity in the 15°C, 20°C, and 25°C groups; (C) Morphology of curly tail of sperm in HOST; C, D: Sperm with intact membrane; E: Sperm with damaged membrane; (D) Acrosome morphology of sperm stained with Coomassie brilliant blue; A: Sperm with intact acrosome; B: Sperm with damaged acrosome. The bars represent the standard error. Letter difference (a-c) means significant difference ($P<0.05$)

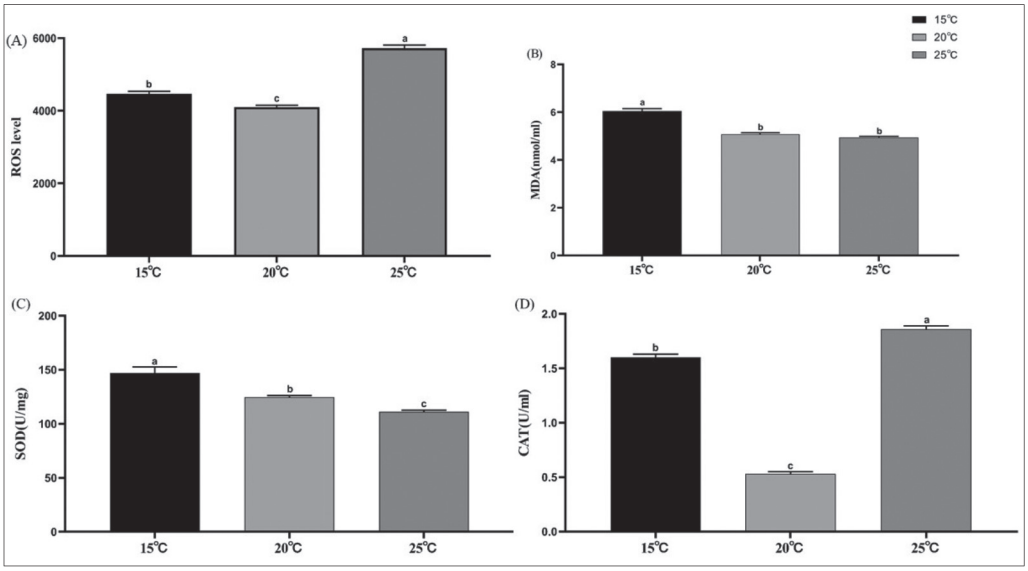

Fig 4. Effects of different temperature on sperm antioxidant parameters. (A) The ROS level of sperm in the 15°C, 20°C, and 25°C groups on the fifth day; (B) The MDA content of semen in the 15°C, 20°C, and 25°C groups on the fifth day; (C) The SOD activity of sperm in the 15°C, 20°C, and 25°C groups on the fifth day; (D) The CAT activity of semen in the 15°C, 20°C, and 25°C groups on the fifth day. The bars represent the standard error. Letter difference (a-c) means significant difference ($P<0.05$)

sperm stored at 25°C had the highest total motility and progressive motility. This may be because in the early stage of semen preservation, higher temperature stimulates sperm activity and metabolism, and lower temperature inhibits sperm movement [11]. This result was consistent with the changes in sperm motility of Hahn et al.[14] in the study of buck sperm. The motility movement speed of buck sperm at 37°C was significantly higher than that at 20°C. The study also found that higher temperatures activate sperm metabolism. For a short time, sperm motility

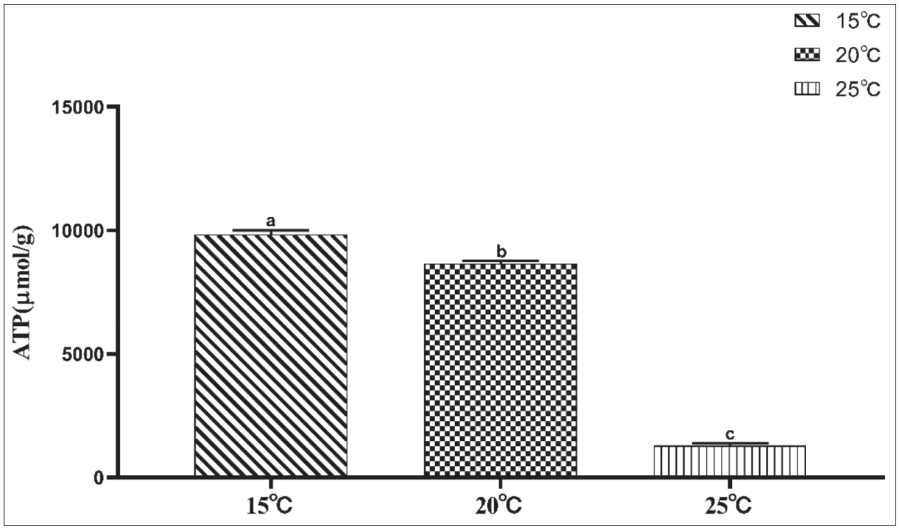

Fig 5. Effects of different temperature on sperm ATP content of Hu ram on the fifth day. The bars represent the standard error. Letter difference (a-c) means significant difference (P<0.05)

at 37°C did not differ from sperm motility at 20°C. This differs from this study in the length of preservation. Within 3~5 days of storage, the sperm total motility and progressive motility saved at 20°C was the highest. Within 6~9 days of storage, the sperm total motility and progressive motility saved at 15°C was the highest. This may be due to the increased respiration and metabolism of sperm at higher temperatures, increased nutrients consumption and low levels of nutrients that cannot meet the metabolic activities of some sperms leading to sperm death [15]. At lower temperatures, sperm motility was restricted and nutrient consumption was slowed down, which can meet the metabolic activity of sperm. This result was consistent with the results of Verstegen et al.[16]'s research on the effect of temperature on dog sperm. High temperature will speed up sperm metabolism, increase nutrient consumption, and reduce sperm total motility. After storage for 5~6 days, the quality of semen stored under various temperature conditions decreased significantly, and the quality of semen stored at higher temperatures changed drastically. This may be due to the fact that in the early stage of semen preservation, a large amount of nutrients was consumed and the remaining nutrients cannot maintain the normal metabolism of sperm. As a result, the death rate of sperm was accelerated and the quality of semen changes dramatically [17]. Storage temperature was an important factor that affects the quality of semen, and it was vital to the survival of sperm [18].

The effective survival time and total survival time of sperm stored at 15°C were the longest. This may be because higher temperature will speed up sperm energy metabolism, faster nutrient consumption, faster bacterial reproduction in semen, and at the same time produce more metabolic waste, which has a certain toxic effect on sperm and reduces the survival time of sperm [19,20]. Within 6~9 days of storage, the VSL, VCL, and VAP of sperms stored at 15°C were the highest. Therefore, VSL, VCL, and VAP were important indicators of sperm motility parameters, which were closely related to fertilization ability [21].

Plasma membrane was the outermost structure of sperm cells and acts as a physiological barrier. The normal physiological functions, capacitation and metabolism of sperm were related to their integrity, and the integrity of the membrane can also indirectly reflect the life and death of sperm [22,23]. The membrane integrity of sperm stored at 15°C and 20°C was the highest within 3~5 days. On the 7th day of storage, the membrane integrity of sperms stored at 15°C was the highest. This result is consistent with changes in sperm total motility. In the first 5 days of preservation, the sperm acrosome integrity rate decreased slowly. On the 7th day of storage, the acrosome integrity rate of sperm stored at 25°C decreased faster. Yeste et al.[24] research shows that osmotic pressure has a great influence on the structure and physiological function of sperm. This study shows that high osmotic pressure has a great effect on the integrity of sperm membrane and acrosome. This study shows that the osmotic pressure is not only related to the solute added to the diluent, but also affected by the type and concentration of ions. In this study, it may be because that after a large number of sperm died, the dead sperm released a large number of ions into the diluent. In this experiment, the acrosome integrity rate of sperm stored at 25°C decreased rapidly. It may be that the osmotic pressure of the diluent changed with the death of a large number of sperm, resulting in a rapid decline in the acrosome integrity rate. The acrosome intact rate in this experiment was directly proportional to the sperm survival time. This result is consistent with the research results of Zhang et al.[25]'s preservation of bovine semen at room temperature. The effective survival time and sperm

motility in the optimal concentration of antioxidant group were the highest, and the sperm acrosome integrity rate was also the highest.

In the process of semen preservation, ROS produced by sperm metabolism has a certain relationship with the decline of sperm quality [26,27]. On the 5th day of storage, sperm stored at 25°C have the highest ROS level. This result was consistent with changes in sperm progressive motility. High concentrations of ROS will damage the integrity of the sperm membrane [28]. This result was consistent with the change of sperm membrane integrity. On the 5th day of storage, the semen stored at 15°C had the highest MDA. This may be due to the low mortality of sperm stored at 15°C, while the other groups have high mortality and low metabolism. This result was consistent with changes in sperm progressive motility and sperm ATP content. The level of sperm ATP content represents the level of mitochondrial activity, and it can also reflect the vitality of sperm, which is positively correlated with fertilization potential [29]. On the 5th day of storage, the sperm SOD activity was highest at 15°C. This may be due to the strong antioxidant system and high enzyme activity of the semen stored at 15°C.

Qiu [30]'s research on the liquid storage of goat semen found that 15°C has a better preservation effect on semen than 5°C and 25°C. In this experiment, compared with 20°C and 25°C, 15°C was the optimum temperature for storage of Hu ram semen at room temperature, which was consistent with the above-mentioned research results.

In conclusion, the most suitable temperature for Hu ram semen to be stored at room temperature is 15°C compared to 20°C and 25°C. The motility of sperm stored at 15°C decreased slowly throughout the preservation process. Sperm stored at 15°C had the highest effective survival time. Preserving semen at this temperature can effectively prolong the survival time of sperm and slow down the rate of sperm apoptosis.

Availability of Data and Materials

All data sets collected and analyzed during the current study are available from the corresponding author (Y. Li) on reasonable request.

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Competing Interests

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

Authors' Contributions

LZ: Conceptualization, methodology, investigation, formal analysis, writing- original draft. TS: Conceptualization, writing-original draft, writing-review & editing. YK, YW and XW: Methodology, investigation. XS: Formal analysis, writing-review & editing. YL: Writing-review & editing, visualization, supervision, project administration. All authors read, revised, and approved the final manuscript.

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