DMEM and FBS as thawing solutions for frozen semen of pigs can improve sperm motility and sow reproductive performance

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Abstract: The purpose of this experiment was to investigate the effects of Beltsville thawing solution (BTS), Androhep, and Dulbecco's modified Eagle medium + Fatal bovine serum (D-F) as diluents of frozen semen on sperm motility and sow reproductive performance. In experiment 1, boar semen was collected and diluted to $2.0 \times 10^9/60$ mL, $1.5 \times 10^9/60$ mL, and $1.5 \times 10^9/40$ mL with Androhep. The appropriate semen dose was determined by postcervical insemination. In experiment 2, boar semen was fumigated with liquid nitrogen and thawed semen was diluted to $1.5 \times 10^9/40$ mL with D-F, Androhep, and BTS, respectively. The effects of above diluents on sperm motility and sow reproductive performance were studied by postcervical insemination. The appropriate semen dose for postcervical insemination was $1.5 \times 10^9/40$ mL. After thawing of frozen semen, sperm motility of semen diluted with D-F was significantly higher than that of semen diluted with Androhep and BTS. There was no significant difference in pregnancy rates among sows via artificial insemination. The litter size of sows using D-F diluted semen was significantly higher than that of sows using Androhep and BTS diluted semen. In conclusion, D-F freezing diluent can improve sperm motility of frozen boar semen after thawing, prolong sperm survival time, and increase sow reproductive performance.

Key words: sow, insemination, frozen semen, thawing, sperm motility

1. Introduction

Artificial insemination (AI) has been used since the early 1930s in pigs. Its true development and widely commercial application did not take place until the 1980s in the pig industry [1,2]. However, according to the report of Didion et al. [3], more than 90% of sows were artificially inseminated with fresh extended semen and less than 1% of sows were inseminated with frozen semen [4]. In pig production, the usage of frozen semen for AI reduced litter size of sows [5,6]. However, it still has many advantages in pig production and breeding processes such as long-term preservation of germplasm resources, the exchange of germplasm resources between home and abroad, no limitation by time and place in insemination of oestrous sows, and so on [7,8]. Moreover, frozen semen can replace fresh semen in in vitro fertilization (IVF) tests [9]. Therefore, many researchers devoted themselves to studying various factors affecting frozen semen, hoping to improve sperm motility of frozen semen and sow reproductive performance, and enable frozen semen to be used in pig production [10].

The methods of AI in pigs include intra-cervical insemination, postcervical insemination, deep intrauterine insemination (deep uterine insemination) and intra-oviductal insemination [1,10]. Because frozen semen has low sperm motility, the post cervical insemination [11–13] and deep uterine insemination [12,14,15] are widely used to improve pregnancy rates and litter sizes.

After thawing of frozen boar semen, sperm motility decreased and the integrity of plasma membrane was destroyed [16]. In order to solve these problems, a series of studies was performed to control freezing rate [17], use microtubules [18,19], change cryogenic media such as adding cholesterol [5], seminal plasma [17,20], antioxidants [21], improve AI technology [14,22] and control sow ovulation time [23]. The production level of frozen semen gradually approached that of fresh semen through the continuous improvement of various factors in pigs [3].

To this end, this experiment first studied the effect of different doses of diluent on sperm motility, and then determined appropriate semen doses required for postcervical insemination. Semen was frozen, was thawed and was diluted to target concentration with Beltsville thawing solution (BTS), Androhep, and Dulbecco's modified Eagle medium + Fatal bovine serum (D-F). The

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postcervical insemination was performed to research the effects of BTS, Androhep, and D-F on sperm motility and sow reproductive performance. This study will provide theoretical and practical basis for the application of frozen semen in pig production.

2. Materials and methods

2.1. Semen collection and dilution
All procedures in this study were approved by the Animal Ethics Committee of Northeast Agricultural University, Harbin, China and were performed with strict adherence to the guide for the Care and Use of Animal for Research Purpose.

Semen was collected from 3 different boars with normal reproductive function (Large Yorkshire, 1.5–2 years old) via gloved hand method. Semen with sperm motility above 0.9 was diluted with commercial extending product Androhep (Minitube, Germany). The semen of each boar was divided into 3 parts for dilution. Semen was extended at sperm density of $2.0 \times 10^9/60mL$, $1.5 \times 10^9/60mL$, and $1.5 \times 10^9/40mL$. The diluted semen was poured into different semen bottles and marked 1, 2, and 3, respectively. The semen bottles were wrapped with towels for 3 layers and were stored in a refrigerator at 17 °C.

2.2. Assessment of sperm motility
Assessment of sperm motility was performed when semen was diluted to 180 to 240 sperms per field. A small drop of diluted semen was dripped into the chamber of a slide. The slide was placed under a microscope with 37 °C thermostat plate. Sperm motility was evaluated objectively using a computer assisted sperm analysis system (CASA) (IVOS Version 12.0, shockexcluding option, Hamilton Thorn Research Beverly, MA, USA) in 5 different fields. The average value of the 5 determinations was sperm motility. Each semen sample was analysed at 24 h, 48 h, 72 h, 96 h, 120 h, and 144 h after dilution, respectively.

2.3. Oestrus detection
The sows were weaned after 28th day of lactation. The weaned sows were identified for estrus regularly every morning and afternoon. When the sows showed symptoms such as decreased appetite, excitement and restlessness, hyperaemia and mucus flow in the genitals, the breeders began to press the back and waist of the sows with their hands. If the sows standed still and tail upturned, that was defined as the rutting sows. Then, the rutting sows were inseminated for the first time 12 h after oestrus and for the second time following 24 h after oestrus.

2.4. Artificial insemination with fresh semen
One hundred and eighty postweaning oestrus sows (Landrace × Large Yorkshire) were divided into 3 groups with 60 pigs in each group. Animals in the A group, B group, and C group were inseminated by postcervical

insemination with semen dose of $2.0 \times 10^9/60$ mL, $1.5 \times 10^9/60$ mL, and $1.5 \times 10^9/40$ mL, respectively. The piglets were weaned after 28 days of sow lactation. And sows that showed oestrus within 3–5 days after weaning were artificially inseminated [1]. The same skilled inseminator of the pig farm operated to reduce human error in the experiment.

2.5. Frozen semen
Semen was frozen according to the methods of Abeydeera et al. [9] and Didion et al. [3] with minor modifications. Briefly, semen quality measurement was performed and sperm density was required to be more than $2 \times 10^8/$ mL. Semen (20 mL) was put into 50 mL centrifuge tube. Then 20 mL of adherent isothermal Androhep (Minitube, Germany) diluent was slowly poured into the centrifuge tube. The homogenous solution was mixed gently at 17 °C for 4 h, then was centrifuged at 1000 g for 8 min. After centrifugation, the supernatant was removed. Coolant I liquid (Androsrar cryoplus, Minitube, Germany) was put in precooling refrigerator at 17 °C and was added to the sperm pellet. Then they were mixed slowly with a pipette tip. The centrifuge tube was transferred into 250 mL beaker (with 150 mL water inside), then the beaker was placed in 4 °C refrigerator to cool. A thermometer was placed in 250 mL beaker which had an auxiliary ice cooling at its bottom to detect the temperature change requirement. The auxiliary ice cooling at the bottom of the beaker was for ensuring a drop in temperature to 4 °C within 90 min. Following the drop on temperature to 4 °C, the coolant II liquid containing glycerol was added and was mixed gently so that the sperm density of 1×10^9 / mL and glycerol content of 2.5% was achieved. The centrifuge tube was put back into 250 mL beaker. The semen was balanced at 4 °C for 0.5 h and was loaded in 0.25 mL tubes. The tubes were placed in fumigation pails (–140 °C to –150 °C) and were transferred into the liquid nitrogen tank after 10 min for storage.

2.6. Thawed semen and assessment of sperm motility
The tubes containing frozen semen were taken out from liquid nitrogen tank with a long tweeze and were quickly transferred into 38 °C water for 30 s to 60 s. Then the tubes were brought out and were quickly dried with a tissue. The semen was slowly diluted to 10 times with BTS, Androhep, and D-F (DMEM + 10% FBS), respectively and was placed in a CO_2 incubator at 37 °C. Sperm motility was evaluated every 15 min.

2.7. Insemination method
Postcervical insemination was performed. The insemination instrument was a disposable vas deferens with cannula. The inseminator wore disposable sterile gloves. One hand separated the labia and the other hand inserted the vas deferens into the vagina, first slightly

upward and then horizontally, until the sponge head at the front of the vas deferens was stuck in the cervix and could not move forward, and then inserted the internal catheter into the uterus for 8–10 cm. The semen bottle was connected to the catheter and was inseminated for 5–7 min.

2.8. Artificial insemination with frozen semen
Thirty postweaning oestrus sows were divided into 3 groups (10 pigs in each group). Postcervical insemination was performed in sows at 12 h and 24 h after oestrus. Thawed semen with sperm motility more than 0.35 was used for dilution with BTS, Androhep, and D-F, respectively. It required that 40 mL semen contained 1.5 billion live sperm. AI was carried out immediately after dilution.

2.9. Statistical analysis
Statistical analysis was performed with SPSS software version 19 (IBM Corp., Armonk, NY, USA). Comparison of percentages were made by chi-square test and others were analysed by ANOVA. Multiple comparisons were performed using the Duncan method. Data were presented as the means ± standard error. P-values < 0.05 were considered significant.

3. Results

3.1. Effect of different volumes of diluent on sperm motility
As shown in Table 1, sperm density decreased from 2.0×10^9/mL to 1.5×10^9/mL in the 60 mL of the diluent which had no significant difference in sperm motility within the same period of time (P > 0.05). The volume of the diluent decreased from 60 mL to 40 mL with 1.5×10^9/mL of sperm density which had no significant difference in sperm motility within the same period of time (P > 0.05). After 144 h of preservation, sperm motility was still above 67.6%. Therefore, 2.0×10^9/60 mL, 1.5×10^9/60 mL, 1.5×10^9/40 mL dilution of three doses had no effect on sperm motility.

3.2. Effect of different volumes of diluent on reproductive rate
Effect of different volumes of diluent on reproductive rate was presented in Table 2. The result showed that sperm density was reduced from 2.0×10^9/mL to 1.5×10^9/mL in 60 mL of the diluent which had no effect on pregnancy rate (91.7% vs 91.7%) and litter size (12.2 vs 12.1) of sows (P > 0.05). The volume of the diluent was reduced from 60 mL to 40 mL with 1.5×10^9/mL of sperm density which had no effect on pregnancy rate (91.7% vs 90%) and litter size (12.2 vs 11.9) of sows (P > 0.05). Therefore, semen dose of 1.5×10^9/40 mL had no effect on reproductive rate.

3.3. Effect of different thawing solutions on sperm motility
The effect of different thawing solutions on sperm motility was significant (P < 0.05) after thawing. Sperm motility was highest in D-F thawing solution, followed by that in Androhep solution and BST solution at each time point (Table 3). Sperm motility decreased with the time and was below 10% until 240 min. In addition, survival time of sperm in different thawing solutions was shown in Table 3. Sperm in D-F solution had a significantly longer survival time, followed by that in Androhep solution and in BTS solution. All thawing solutions maintained over 30% survival rate within the first 30 min. There was about 1.3% surviving sperm in BTS while D-F and Androhep thawing solutions had 7.7% and 4.8% surviving sperm after 240 min, respectively. It can be seen that the diluent composed of D-F can improve the sperm motility of thawed semen and prolong survival time of sperm.

3.4. Effect of different thawed semen diluents on reproductive performance
Table 4 showed the effect of different thawed semen diluents on sow reproductive performance. The pregnancy rate, litter size, and alive litter size of sows inseminated with frozen semen diluted with D-F thawing solution were significantly higher (P < 0.05) than those of sows inseminated with frozen semen diluted with BTS and Androhep thawing solution.

4. Discussion
Compared with the semen of bull and ram, boar semen is large in quantity and low in sperm density. Therefore, boar semen must be centrifuged for cryopreservation. After thawing frozen semen, it needs to be diluted again before it can be used to inseminate. Every step in the

Table 1. Effect of different volumes of diluent on sperm motility (%, billion, n = 10).

	24 h	48 h	72 h	96 h	120 h	144 h
2.0/60 mL	85.0 ± 2.2[a]	83.7 ± 2.5[a]	83.3 ± 2.5[a]	79.3 ± 3.1[a]	76.7 ± 2.9[a]	68.3 ± 4.6[a]
1.5/60 mL	83.8 ± 2.4[a]	81.1 ± 3.0[a]	79.3 ± 3.5[a]	77.2 ± 2.8[a]	76.6 ± 3.9[a]	67.6 ± 4.7[a]
1.5/40 mL	84.5 ± 2.8[a]	82.3 ± 2.5[a]	82.1 ± 2.7[a]	80.6 ± 1.8[a]	77.8 ± 3.6[a]	69.9 ± 4.9[a]

Means with the same superscript along the same column were not statistically different (P > 0.05).

Table 2. Effect of different volumes of diluent on reproductive rate (postcervical, billion, n = 4).

Group	Number of sows	Pregnancy rate	Average litter size	Alive litter size
A	60	91.7[a] (55/60)	12.2[a] (671/55)	10.9[a] (601/55)
B	60	91.7[a] (55/60)	12.1[a] (668/55)	10.9[a] (602/55)
C	60	90[a] (54/60)	11.9[a] (654/54)	10.7[a] (579/54)

A group was inseminated with semen dose of 2.0×10^9/60 mL. B group was inseminated with semen dose of 1.5×10^9/60 mL. C group was inseminated with semen dose of 1.5×10^9/40 mL. Means with the same superscript along the same column were not statistically different (P > 0.05).

Table 3. Effect of different thawing solutions on sperm motility (%, n = 10).

	0 min	15 min	30 min	60 min	120 min	240 min
BTS	40.2 ± 3.7^a	38.7 ± 3.9^a	22.3 ± 4.1^a	10.4 ± 4.2^a	4.2 ± 2.7^a	1.3 ± 2.4^a
Androhep	43.3 ± 3.5^b	41.2 ± 2.8^b	30.6 ± 3.4^b	16.5 ± 4.4^b	10.9 ± 3.3^b	4.8 ± 2.9^b
D-F	48.5 ± 3.9^c	45.5 ± 3.1^c	34.8 ± 3.5^c	22.8 ± 4.7^c	13.8 ± 2.9^c	7.7 ± 2.8^c

0 min means that BTS, Androhep and D-F were added to the semen immediately after thawing, and recorded as 0 min. Means with different superscripts along the same column were statistically different (P < 0.05). Note: D-F, DMEM + 10% FBS.

Table 4. Effect of different thawed semen diluents on sow reproductive performance (n = 3).

	Number of sows	Pregnancy rate	Litter size	Alive litter size
BTS	10	80[a] (8/10)	7.6[a] (61/8)	7.5[a] (60/8)
Androhep	10	80[a] (8/10)	7.8[a] (62/8)	7.5[a] (60/8)
D-F	10	90[b] (9/10)	9.2[b] (83/9)	8.9[b] (80/9)

Means with different superscripts along the same column were statistically different (P < 0.05).

process of semen freezing has an effect on sperm motility of frozen semen and sow reproductive performance after insemination. Therefore, this experiment studied the influence factors in the process. When semen is diluted at different densities, sperm motility and survival time of sperm are different. The result obtained in this experiment showed that semen extended in 40 mL and 60 mL had no significant effect on sperm motility. Semen with sperm count of 1.5×10^9/mL can be extended from 40 mL to 60 mL without any significant effect on sperm motility, providing the advantage of effective use of semen for insemination. Because sperm motility of frozen semen is low after thawing, the postcervical insemination [11–13] and deep uterine insemination [12,14,15] are widely used to improve conception rate and litter size. Deep uterine insemination

can deliver sperm to proximal 1/3 position of uterus horn [24]. Less sperms were consumed to achieve normal reproductive level, which are beneficial to the use of frozen semen. In this experiment, postcervical insemination was employed, the volume of semen was reduced from 60 mL to 40 mL, and the number of effective sperm was reduced from 2 billion to 1.5 billion, which can fully meet the needs of production and lay a technical foundation for the further use of frozen semen. The study of Watson and Behan showed that 1 billion sperms could reach normal level of production via postcervical insemination [25]. The results of Roberts and Bilkei indicated that 1 billion sperms caused the decrease in litter size of sows via post-cervical insemination [26]. Effective sperm count used in this experiment was 1.5 billion and there was no test whether 1 billion sperms could

reach normal production level. Survival time of sperms and ova in the reproductive tract of sows is limited, so appropriate time of AI is an important factor. Since sperm motility of frozen sperm is low, survive time of sperm in the reproductive tract of sows will be shortened. It is usually necessary to inseminate at 4–8 h before ovulation [27] or use reproductive hormones to control the ovulation time of sows [28,29] for real-time insemination. In this experiment, sows were naturally oestrus and not treated with hormones. Therefore, ovulation time and physiological status of sows may have some influence on the experiment results.

A different pattern was observed when D-F, Androhep, and BST were used to study postthawing sperm motility. A significant difference was found in sperm motility after thawing. Androhep and Androstar were commonly commercial semen extenders. However, the result obtained in this study suggested that D-F was better than Adrohep in preserving semen to obtain high sperm motility after thawing. The same trend was shown in sperm survival time. It may be that some components of fatal bovine serum (FBS) in thawing solution affected sperm activation, sperm motility, and metabolism. FBS contains many bioactive substances, which are commonly used nutrients and antifreeze protectors in culture and freezing of embryo and cell [30]. Therefore, it is necessary to further verify the effects of FBS on sperm motility, survival time, metabolic mode, and fertilization ability at room temperature, low temperature, and ultralow temperature. In addition, whether FBS had biological risk after being imported into sow uterus, whether it was suitable for long-term use or BSA can replace FBS were needed further study.

After freezing and thawing of boar sperm, sperm motility is related to the composition of thawing solution [31,32,33]. When direct insemination is concerned with physiological state of female animal, these factors will affect sperm motility [34–37]. Jose et al. added 2 IU oxytocin, 5 μg lecirelin, and 2 mM caffeine to semen diluent 15 min before AI, which significantly improved conception rate and litter size of sows [38]. Therefore, the physiological characteristics of sperm are very complex and sperm motility is influenced by many factors. In addition, sperm motility after thawing does not truly reflect sperm ability to be fertilized. It is still not known whether they can initiate movement when they enter reproductive tract of dam. Since in our experiment, it was found that the same tube of semen was thawed and then diluted with different thawing solutions, sperm motility was different. Therefore, we believed that although motor ability and fertilization ability of sperms were highly correlated, we cannot rule out sperms that were not moving under the microscope. Without exercising under environmental conditions in female reproductive tract, there was no ability to fertilize.

5. Conclusion
DMEM and FBS diluents can increase sperm motility of frozen boar semen, prolong survival time of sperms, and increase sow reproductive performance.

Conflicts of Interest
The authors declare that they have no conflicts of interest.

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