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Assessment of the Quality of Frozen-Thawed Semen or Epididymal Sperm in Three Native Vietnamese Pig Breeds

Van Khanh Nguyen[1*], Huong Thu Thi Vu[1], Huong Le Thi Nguyen[1], Huong Thi Nguyen[1], Au Thi Hoang[1], Dat Van Le[1], Nhung Tuyet Thi Nguyen[1], Hieu Trung Phan[1], Yen Kim Thi Pham[1], Son Phan Le[1], Quynh Anh Le[1], Lan Doan Pham[2], Minh Quang Luu[3], Muoi Thi Nguyen[4]

[1]Key Laboratory of Animal Cell Biotechnology, National Institute of Animal Science, Hanoi, Vietnam
[2]National Institute of Animal Science, Hanoi, Vietnam
[3]Ministry of Science and Technology, Hanoi, Vietnam
[4]Animal Experiments and Domestic Animal Conservation Center, National Institute of Animal Science, Hanoi, Vietnam
Email: *cotihin@gmail.com

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Abstract

Our aim was to evaluate the quality of ejaculated and epididymal frozen-thawed pig sperm of endangered Vietnam native pig breeds. Ejaculated sperm was collected from live boars and epididymal sperm was collected from slaughtered boars of the *MuongTe*, *Kieng Sat* and *Co BinhThuan* breeds and frozen in 0.25 ml straws using a protocol established earlier for modern pig breeds. We evaluated the sperm quality after thawing in terms of motility and rates of viable and abnormal spermatozoa. Our results revealed that the sperm motility and rates of viable and abnormal frozen-thawed sperm were >30%, >44%, and <14%, respectively. The origin of sperm had an effect on the production of pig embryos *in vitro*. In the *Co BinhThuan* breed, ejaculated sperm generated higher cleavage, blastocyst and hatching rates than did the epididymal sperm (60.11% vs 56.02%, 17.23% vs 14.31%, 3.78% vs 2.34%, respectively, $P < 0.05$). Although no difference in cleavage rate, blastocyst formation rate and the average number of cells/blastocysts, the hatching blastocyst rate was different between the breeds ($P > 0.05$). In the *Co BinhThuan* breed, the rate of pregnancy of ejaculated groups was similar to that of the epididymal group. In conclusion, the ejaculated and epididymal sperm of native Vietnamese pigs were successfully frozen. We succeeded in creating embryos *in vitro* and pregnant pigs after artificial insemination from frozen-thawed semen in three native Vietnamese pig breeds for the first time. The use of the ejaculated sperm improved the production of native pig embryos *in vitro* efficiency.

Keywords

Native Vietnamese Pig, *In Vitro* Porcine Embryo, Epididymal, Ejaculate, Artificial Insemination

1. Introduction

Vietnam has a high biodiversity with many different native livestock breeds, especially indigenous pig breeds [1]. Although native Vietnamese pigs are characterized by small size and low productivity, they have valuable features as well as premium meat quality, good adaptability to harsh raising conditions or poor feeding, and good disease resistance. These characteristics make them suitable for breeding in remote, uninhabited areas. However, recently, the numbers of native Vietnamese pigs have been decreasing dramatically due to globally standardized economical pig production, this leads to the increasing use of modern breeds (*i.e.* Landrace, Large White and Duroc) or crossbreeds between them. Furthermore, recent epidemics of African swine fever perished a great number of Vietnamese native pigs [2], which were mainly raised in remote rural areas where disease control was difficult. The loss of these native Vietnamese pigs poses a serious threat to the reduction of porcine genetic diversity in Vietnam. Therefore, *in vitro* conservation of native pig breeds using assisted reproductive techniques such as cryopreservation of sperm is urgently needed since it enables the preservation of genetic materials safe from diseases.

Sperm cryopreservation is also a useful tool for breeding programs since it enables the long-term storage of sperm from high-performing boars. Thus, it can be served as the future improvement of native Vietnamese breeds. For these reasons, sperm cryo-conservation is the necessary basis to establish the genome resource banks, that is, organized repositories of frozen biomaterial.

Cryopreservation of porcine sperm obtained either as ejaculated semen or epidiymal sperm by slow freezing in 0.25 or 0.5 ml plastic straws has been established in modern breeds [3]. However, in pigs, artificial insemination with frozen sperm is still challenging [4]. For the utilization of frozen boar sperm, the *in vitro* production of embryos by *in vitro* fertilization (IVF) of oocytes is an important technique since it can generate a large number of high-quality embryos that can be transferred to surrogate mothers to obtain piglets under controlled conditions [5]. Sperm freezing and *in vitro* production of porcine embryos have been successfully established in modern pig breeds such as Landrace, Large White or Duroc [6] but only recently adapted for the utilization of epididymal sperm in only one native Vietnamese breed, the *Ban* [6] [7] [8].

In Vietnam, indigenous pig breeds such as the *Co Binh Thuan*, *Muong Te* and *Kieng Sat* are endangered animals that need to be preserved according to Decree 13/2020/ND-CP of the Vietnamese Government [9]. Therefore, freezing of epidiymal sperm and ejaculated semen plays an important role in the preservation

of these breeds and in establishing animal gene banks to preserve them. Therefore, the aim of the present study was to establish the protocols for freezing ejaculated and epidiymal sperm in these pig breeds and assess their feasibility of producing embryos by IVF and subsequent embryo culture. First, we applied the protocol originally established for modern breeds and evaluated sperm quality parameters such as motility and rates of viable and abnormal spermatozoa. Then, we compared the feasibility of frozen/thawed semen and epidiymal sperm for embryo production and the pregnancy rate after artificial insemination of *Co BinhThuan* breed. Finally, we compared the efficacy of frozen-/thawed semen in three native Vietnamese breeds (*Co BinhThuan*, *MuongTe* and *Kieng Sat breeds*) for *in vitro* embryo production by IVF.

2. Materials and Methods

2.1. Collection Spermatozoa by Ejaculation Method

Ejaculated semen was collected from *Kieng Sat*, *MươngTe*, *Co BinhThuan* boars (7 - 10 months old) according to SATREPS/Vietnam protocol (2020) [10]. When the boar is mounted on the dummy, sow the spiral end of the penis with hands (gloved or bare, must be cleaned, dried and warmed up). To allow the boar to thrust through a clenched hand several times before applying a pressure, a hand pressure was applied to the spiral part of the penis to imitate the estrous sow's cervix, stimulation ejaculation. When the penis is locked in the hand and the boar feels relaxed, a four-phase ejaculation follows in a few seconds, taking 5 to 10 minutes to completion. The first phase, called the pre-sperm fraction, contains clear seminal fluid, some gel, and dead sperm cells and is heavily contaminated with bacteria. It should not be collected. The next phase is the rich fraction sperm, easily recognized by its creamy-white color, although only 30 - 40 ml in volume contains a high density of spermatozoa. The third fraction, which is grayish with a lower density of spermatozoa, accounts for 50 - 70 ml of the collection. The fourth phase or post-sperm fraction provides a large semen volume peculiar. Up to the volume of 100 - 200 ml clear seminal plasma that is free of spermatozoa and gel is secreted from the accessory glands.

Because spermatozoa are very sensitive to rapid temperature changes, require a warm and dry collecting flask to safeguard semen fertility. In order to collect semen from boars, we used glass tubes (Sterile, dark colored, 300 - 500 ml) and semen filter paper. After ejaculation, semen were divided into the tubes and kept at 32˚C in a thermos bottle and transferred to the lab, immediately.

2.2. Collection Spermatozoa by Epididymis Method

The collection spermatozoa from epididymis method were described by Kikuchi *et al.* (1998) [11]. Testes with attached epididymis were obtained from *MươngTe*, *Kieng Sat* and *Co BinhThuan* boars. Immediately, after removal from the scrotums, the testes were placed into plastic bags with sterile isotonic saline solution at room temperature and transferred to the laboratory within 1 h. In the labora-

tory, the epididymides were dissected and separated from the testis. Each cauda epididymis was dissected free, rinsed with 0.9% saline and placed into a 100 mm Petri dish. Caudae epididymides were held with forceps, and multiple incisions were made in the tubuli with a bistoury. Then, the spermatozoa were extruded from the caudaepididymidis by air pressure from a syringe and collected using 30 ml collecting solution. Sperm were diluted with a medium during storage at 15˚C for 2 - 3 hours.

2.3. Freezing Sperm and Semen

Collected semen and epidiymal sperm was frozen using the method described in the method of Kikuchi *et al.* (1998) [11].

Before freezing, collected sperm were placed on prewarmed glass sildes at 37˚C, and observed under a stereo microscopy for subjective evaluation of motility and morphological and evaluated the motility and morphology under a stereo microscope. Semen from boars which have more than 80% of total motility and 80% of normal spermatozoa were used. A sperm having an oval shaped head, an intact midpiece and an uncoiled single tail will be considered as a normal and healthy sperm. Sperms with normal morphology are able to swim well and in a straight line. Then, sperm concentration in samples was determined with a Neubauer chamber, with a dilution of 10 µl of semen to 990 µl of water. Thereafter the collected semen or epidiymal sperm was diluted to a ratio of 1:1 with collection medium prewarmed at 32˚C. Collection medium including 330 mM D-Glucose, 12.8 mM Sodium citrate, EDTA 9.9 mM, 14.3 mM Na-Bicarbonate, Penicillin (1000 unit/ml), Streptomycin (1 mg/ml).

After dilution with the collection medium, the semen was placed at 15˚C for 2 hours and then centrifuged at 3000 r/minute for 10 minutes at 15˚C. Then the supernatant was removed, and the pellet was stepwise resuspended in NSF-I extender to approximately 15 - 25 ml depending on the size of the sperm pellet. After dilution with NSF-I, the sperm was placed at 5˚C for an additional 2 hours. Thereafter, all steps were performed at 5˚C. Motility was verified again, and the sperm was diluted to 1:1 with NSF-II extender to a final volume of 30 - 50 ml. Then sperm was loaded to 0.25 ml plastic straws (Minutube) within 10 minutes which were immediately layered in the vapor above liquid nitrogen and kept there for 10 minutes. Then the frozen straws were moved under liquid nitrogen and stored there until use. For the final testing of motility, a frozen sperm straws from each lot was thawed by directly moving it with tweezers in a water bath at 37˚C for 20 seconds. Subsequently, the straw was wiped dry, opened with scissors and its content was placed on a microscope slide heated to 37˚C and evaluated for the parameters previously described for the semen immediately after collections.

2.4. Oocytes Collection and *in Vitro* Maturation

In brief, we obtained pig ovaries from 6 - 8 months old crossbred gilts (Landrace

× Large White) at a local slaughterhouse and transferred to the laboratory within 5 h in saline at 35˚C - 37˚C. The ovaries were washed 3 times in Dulbecco's Phosphate Buffer Saline (DPBS; Sigma-Aldrich Corp., St. Louis, MO, USA) supplemented with 0.1 mg/ml streptomycin sulfate (Sigma-Aldrich) and 100 units/ml penicillin G potassium (Sigma-Aldrich) at 37˚C and processed as follows. Cumulus-oocyte complexes (COCs) were collected by aspiration (at least 1 mm in diameter) into a collection medium consisting of Medium 199 (with Hank's salts; Sigma-Aldrich) supplemented with 5% fetal bovine serum (FBS, Gibco; Invitrogen Corp., Carlsbad, CA, USA), 20 mM HEPES (Dojindo Laboratories, Kumamoto, Japan), and antibiotics 100 units/ml penicillin G potassium (Sigma-Aldrich) and 0.1 mg/ml streptomycin sulfate (Sigma-Aldrich)] in 60 mm petri dishes (Falcon 351007, Thomas Scientific, NJ, USA). COCs were collected under a stereo microscope. Oocytes with no apparent signs of lysis, having evenly granulated cytoplasm and at least 3 intact layers of cumulus cells were used.

Oocytes were cultured in a maturaion (porcine oocyte medium, POM) [12]. The POM was supplemented with, 10 ng/ml epidermal growth factor (EGF, Sigma-Aldrich), 10 IU/ml eCG (Serotropin; ASKA Pharmaceutical Co., Ltd., Tokyo, Japan), and 10 IU/ml hCG (500 units; Puberogen, Novartis Animal Health, Tokyo, Japan) throughout the entire IVM to the report of Van Khanh *et al.* (2021) [13]. The IVM medium was supplemented with 1 m Mdibutyrylc AMP (dbcAMP; Sigma) for the first 22 h of IVM to synchronise oocyte maturation. IVM culture was performed in 4-well dishes (NuncMultiDishes, Thomas Scientific) in 500-µl droplets of IVM medium covered by paraffin oil (Paraffin Liquid; NacalaiTesque) for 22 h in a condition of 5% CO_2, 5% O_2, and 90% N_2 at 39˚C. The COCs were subsequently cultured in the maturation medium without dbcAMP for an additional 22 - 24 h under the same atmosphere. Thirty to 50 COCs were cultured in each well.

2.5. *In Vitro* Fertilization (IVF) and *in Vitro* Embryo Culture (IVC)

In vitro fertilization and *in vitro* embryo culture were performed according to the method of Kikuchi *et al.* (2002) [14]. The medium used for IVF was Pig-FM (Suzuki *et al.*, 2002) [15] containing 90 mmol/L NaCl, 12 mmol/L KCl, 25 mmol/L $NaHCO_3$, 0.5 mmol/L NaH_2PO_4, 0.5 mmol/L $MgSO_4$, 10 mmol/L sodium lactate, 10 mmol/L HEPES, 8 mmol/L $CaCl_2$, 2 mmol/L sodium pyruvate, 5 mmol/L caffeine and 5 mg/ml BSA (fraction V; Sigma). Before IVF, the outer layers of cumulus cells were removed from COCs after a short treatment with 0.1% hyaluroniadase (w/v). Then, the oocytes were washed three times in IVF medium and transferred to 90 µl IVF drops (10 - 20 oocytes/IVF drop) covered by mineral oil (Sigma). Frozen-thawed spermatozoa was placed in 7 ml of sperm washing medium (M199-Sigma, pH adjusted to 7.8) and centrifuged for 2 min at 2000 rpm. Then the pellet was re-suspended with 50 µl sperm washing medium and the motility of sperm was assessed subjectively under a stereo microscope. Only sperm samples with at least 30% motility were used for IVF. Then, 100 µl

of the sperm suspension was transferred to a 30 mm Petri dish, covered by mineral oil and incubated at 37˚C for 15 min (Kikuchi *et al.*, 1998). Thereafter, the sperm was stepwise diluted with IVF medium in a 4-well plate to achieve a concentration of 1×10^6/ml. Then, 10 µl of the sperm dilution was introduced into 90 µl IVF droplets containing oocytes to achieve the final concentration of 1×10^6/ml. The IVF drops were the incubated at 38.5˚C under 5% CO_2, 5% O_2 in humidified air for 6 hours. At 6 h after IVF, the spermatozoa and cumulus cells were removed from the surface of the zonapellucida by gentle pipetting with a fine glass pipette. After that, presumptove zygotes were cultured in 500 µl drops of PZM3 medium in 4-well dishes at 38.5˚C, 5% CO_2 in humidified air.

2.6. The Insemination Process

The insemination process was performed according to the method of Worwod (2007) [16]. The sows should be as acim and relaxed as possible before, during and after the insemination process. Clean the vulva of the sow or gilt by a damp cloth or paper towel. The semen is pulled into the sow by the rod. The reds are desgned to "lock" into the cervix of the sow with counterlockwise threads on the tip or rods with a rounded foam tip. Before gently inserting the rod into the vulva lubricate the tip with semen or a little lubricating jelly, and angle the rod tip upward. The rod with counterlockwise inserted the cervix. The rod with a rounded foam tip is inserted like threaded rod, but don't need to be rotated, gently push until the foam tip catch in the foilds of the cervix. When the sow "accepts" the semen, insert bottle removed the tip into the end of the rod and gentle pressure, then the semen will begin to flow into the sow. Remove the bottle from the rod when it is becoming empty and add some air into the bottle, then reattach it and gently force the last of the semen from the rod and rotate the rod clockwise and withdraw it. Check the sow for standing heat 12 hours and 24 hours after last insemination, the best is the sow stops standing within 12 hours of insemination, the sow still standing maybe we inseminated too soon. The sows were checked for pregnancy by trans-abdominal ultrasound examination 28 days after inseminated. Pregnant sows were confirmed pregnant on Day 60.

2.7. Experimental Design

2.7.1. Experiment 1: Evaluation of Basic Sperm Quality Parameters before and after Freezing Epididymal and Ejaculated Sperm

In this experiment, we investigated the effects of sperm freezing on sperm by comparing basic sperm quality parameters before after freezing of ejaculated semen and epidiymal sperm in 3 native Vietnamese breeds. In each of the *MuongTe*, *Kieng Sat* and *Co BinhThuan* breeds a total of 10 boars were used. The boars were 10 months-3 years old, healthy, without malformations, and with a body weight normal for the breed average. In each breed, 5 boars were used to collect ejaculated semen and another 5 boars were sacrificed for the collection of epididymal sperm. Then collected sperm was frozen as described above. In each boar, sperm quality parameters including average volume, sperm concentration, per-

centages of motile, viable and morphological normal sperm were evaluated before and after freezing as described above.

2.7.2. Experiment 2: Influence of Sperm Origin (Epididymal or Ejaculated) on the Production of Pig Embryos *in Vitro* Using Frozen-Thawed Sperm

IVF was performed with either epidiymal or ejaculated frozen thawed spermatozoa *Co BinhThuan* pig as described earlier. The cleavage, blastocyst and hatching rates were compared among groups. Six biological replications were performed.

2.7.3. Experiment 3: Influence of Sperm Origin (Epididymal or Ejaculated) on the Artificial Insemination Using Frozen-Thawed Sperm

AI was performed with either epididymal or ejaculated frozen thawed spermatozoa *Co BinhThuan* pig as described earlier. The pregnant rate was compared among groups. This experiment using 10 *Co BinhThuan* sows.

2.7.4. Experiment 4: Influence of Breed on the Production of Pig Embryos *in Vitro*

Based on the results of experiment 2, IVF was performed using ejaculated frozen thawed spermatozoa collected from in *MuongTe*, *Kieng Sat* and *Co BinhThuan* pigs as described above. Cleavage, blastocyst and hatching rates were compared among groups. Six biological replications were performed.

2.8. Statistical Analysis

All data were expressed as mean ± SEM values and the significant difference was checked by the ANOVA. $P < 0.05$ was defined as the significance level.

3. Results

3.1. Quality Parameters of Pig Semen Collected from Epididymis and Ejaculate before and after Freezing (Experiment 1)

The quality of epididymis and ejaculated semen were assessed based on the average of the collected semen volume, sperm motility, sperm concentration, viable sperm and abnormal sperm (Table 1). The average volume of epididymal sperm was lower than that of ejaculation (from 2.98 to 3.98 ml vs from 71.32 ml to 79.24 ml, respectively). The quality of epididymal and ejaculated sperm of *MuongTe*, *Kieng Sat*, and *Co BinhThuan* pigs after freezing and thawing were based on percentages of motile sperm, viable sperm and abnormal sperm (Table 2). In this study, there was no significant difference in the sperm motility, viable sperm and abnormal sperm rates between the epididymis and ejaculation group (Table 2, $P > 0.05$).

3.2. Influence of Sperm Orginin (Epididymal or Ejaculated) on the Production of Pig Embryos *in Vitro* Using Frozen-Thawed Sperm (Experiment 2)

We compared the feasibility of embryo fertilized with frozen-thawed epididymal

Table 1. Sperm characteristics of *MuongTe*, *Kieng Sat* and *Co BinhThuan* pig breeds after collection by epididymis or ejaculation method.

Sperm characteristics	Muong Te		Kieng Sat		Co BinhThuan	
	Epididymis	Ejaculate	Epididymis	Ejaculate	Epididymis	Ejaculate
Semen volume (ml)	3.91 ± 1.02	79.24 ± 1.56	2.98 ± 1.12	71.32 ± 1.34	3.98 ± 2.19	75.48 ± 2.08
Sperm motility (%)	77.96 ± 2.16	78.94 ± 2.06	79.01 ± 2,15	77.98 ± 1.76	78.42 ± 1.32	79.06 ± 2.42
Sperm concentration (million/ml)	9610.64 ± 102.32	287.02 ± 3.84	9635.11 ± 143.12	281.15 ± 3.21	9721 ± 98.56	290.32 ± 2.56
Viable sperm (%)	85.07 ± 1.97	83.26 ± 2.67	82.37 ± 2,08	80.98 ± 1.96	83.65 ± 2.21	82.96 ± 2.12
Abnormal sperm (%)	10.08 ± 2.08	10.78 ± 2.68	10.98 ± 2,33	9.02 ± 2.34	9.12 ± 1.78	9.65 ± 1.94

Five replicates were performed. Data are presented as means ± SEM.

Table 2. Characteristics of epididymal and ejaculated sperm in *MuongTe*, *Kieng Sat* and *Co BinhThuan* pig breeds after freezing and thawing.

Sperm characteristics	Muong Te		Kieng Sat		Co BinhThuan	
	Epididymis	Ejaculate	Epididymis	Ejaculate	Epididymis	Ejaculate
Sperm motility (%)	33.76 ± 2.58	32.42 ± 1.87	31.65 ± 2.76	34.88 ± 2.89	32.87 ± 2.36	35.81 ± 1.34
Viable sperm (%)	44.96 ± 2.01	45.24 ± 1.14	45.48 ± 1.69	45.98 ± 2.09	44.86 ± 2.45	45.25 ± 1.93
Abnormal sperm (%)	13.34 ± 1.54	11.74 ± 2.31	12.99 ± 1.62	11.45 ± 2.65	12.08 ± 2.37	11.96 ± 2.08

Five replicates were performed. Data are presented as means ± SEM.

and ejaculated sperm in the *Co BinhThuan* breed. Based on the percentages of oocytes cleavage, blastocyst formation, blastocyst hatching and the cell number of blastocysts as described above. The results are summarized in Table 3. The rates of oocytes cleaved, blastocyst formation, and hatching blastocyst of ejaculated groups were significantly higher than that of the epididymal groups (60.11% vs 56.02%, 17.23%vs 14.31%, 3.78%vs 2.34%, respectively, $P < 0.05$). However, there was no significant difference in the average cell number of blastocysts among the groups (50.18 vs 50.02, respectively, $P > 0.05$).

3.3. Influence of Sperm Prigin (Epididymal or Ejaculated) on the Artificial Insemination Using Frozen-Thwaed Sperm

We compared fertility of frozen-thawed epididymal and ejaculated sperm in the *Co BinhThuan* breed based on the pregnancy rate at Day 60 after aritifical insemination. The results are summarized in Table 4. The rate of pregnancy of ejaculated group was similar to that of the epididymal group (60% vs 60%).

3.4. Influence of Breed on the Production of Pig Embryos *in Vitro* (Experiment 3)

In this experiment, we used the frozen-thawed sperm that collected by ejaculation method of *MuongTe*, *Kieng Sat* and *Co BinhThuan* pigs to do IVF. The results are showed in Table 5.

Table 3. Effect of sperm origin on the *in vitro* production of pig embryos in the *Co BinhThuan* breed.

	Epididymis	Ejaculate
Cleavage (%, mean ± SEM)	1042/1864 (56.02 ± 1.96)[a]	1129/1889 (60.11 ± 3.65)[b]
Blastocyst formation (%, mean ± SEM)	265/1864 (14.31 ± 2.11)[a]	321/1889 (17.23 ± 2.66)[b]
Hatching blastocyst (%, mean ± SEM)	42/1864 (2.34 ± 1.68)[a]	71/1889 (3.78[b] ± 2.42)[b]
Average number of cells/blastocyst	50.02 ± 2.89	50.18 ± 2.97

Six replicates were performed. Percentage values are presented as mean ± SEM. Different superscripts (a, b) denote a significant difference in the same row (P < 0.05).

Table 4. Effect of sperm origin on the artificial insemination in the *Co BinhThuan* breed.

	Epididymis	Ejaculate
The number of sows	5	5
Pregnant (%)	3/5 (60%)	3/5 (60%)

Table 5. Effect of breed of ejaculated frozen-thawed sperm on the production pig embryos *in vitro*.

	Muong Te	Kieng Sat	Co BinhThuan
Cleavage (%, mean ± SEM)	1074/1825 58.96 ± 2.23	1169/1915 61.38 ± 3.82	1129/1889 60.11 ± 3.65
Blastocyst formation (%, mean ± SEM)	328/1825 18.02 ± 2.67	365/1915 19.14 ± 1.97	321/1889 17.23 ± 2.66
Hatching blastocyst	46/1825 2.59[a] ± 1.24	57/1915 3.01[ab] ± 1.68	71/1889 3.78[b] ± 2.42
Average number of cells/blastocyst	50.36 ± 2.98	51.02 ± 3.42	50.18 ± 2.97

Six replicates were performed. Percentage values are presented as mean ± SEM. Different superscripts (a, b) denote a significant difference in the same row (P < 0.05).

There was no significant difference in cleavage rate, blastocyst formation rate and the average cell number of blastocysts among the groups (Table 5, P > 0.05). However, the hatching blastocyst rate was significantly different between the groups. The percentage of hatching blastocysts of *Co BinhThuan* group was higher than *MuongTe* group (3.78% versus 2.59%, P < 0.05, respectively) but no difference between *Co BinhThuan* and *Kieng Sat* groups (respectively, 3.78% vs 3.01%, P > 0.05) or *MuongTe* and *Kieng Sat* groups (respectively, 2.59% vs 3.01%, P > 0.05).

4. Discussion

Because the semen obtained by ejaculation contains sperm and also a large amount

of non-sperm, while semen from the epididymis is mostly sperm. In addition, the semen collected from the epididymis contains a small amount of non-sperm, therefore, the sperm concentration of the epididymis group was higher than that of the ejaculated group. The sperm concentration of the epididymis group in this study was higher than that reported by Nguyen *et al.* (2015) [6]. According to Nguyen *et al.* (2015) [6], the sperm concentration collected from the epididymis of Ban pig was only 1240 million sperm/ml.

Sperm motility and viable sperm are important parameters affecting the quality and ability of sperm to fertilize. In our study, the sperm motility and viable sperm of the ejaculated group and epididymis groups in MuongTe, Kieng Sat, and Co BinhThuan pigs were >77% and >80%, respectively (**Table 1**). The sperm motility of MuongTe, Kieng Sat, and Co BinhThuan pigs was higher than that reported by Nguyen *et al.* (2015) [6]. According to Nguyen *et al.* (2015) [6], the sperm motility of Ban pigs was 70.5%. The difference between the study results may be due to semen extraction technique, breed differences, quality and origin of boars.

The rate of viable sperm after freezing and thawing in our study was higher than that reported in Ban pigs by Nguyen *et al.* (2015) [6]. According to Nguyen *et al.* (2015) [6], the percentage of viable sperm frozen-thawed Ban pig was 31.9%. The difference between the study results may be due to the quality of the sperm before freezing. The rate of motility of sperm before freezing in our study was higher than that reported in Ban pigs by Nguyen *et al.* (2015) [6] (>77% vs 70.5%, respectively).

In our study, sperm motility, viable sperm and abnormal sperm after thawing were lower than that of before freezing (**Table 1**). Our research results are also consistent with the report of Watson (2000) [17], Waterhouse *et al.* (2006) [18] and Pamungkas *et al.* (2012) [19]. According to Watson (2000) [17] and Waterhouse *et al.* (2006) [18], cryopreservation reduces sperm viability after thawing (less than 50% of the spermatozoa survive) and their fertilizing is affected. Freeze/thaw processes of boar semen cause damage to the membrane, mitochondria, motility, and viability of sperm [17]. Pamungkas *et al.* (2012) [19] showed that after thawing, the percentage of viable spermatozoa decreased and morphologically abnormal spermatozoa increased. Sudden temperature changes during cryopreservation cause protein and lipid substitution, affecting the permeability and functionality of the plasma membrane and acrosomal [20]. When temperatures are lower than 5˚C, lateral movement of membrance phospholipids is usually restricted. Temperature changes lead to membrane lipids being restructured, and integral proteins in the plasma membrane becoming irreversibly clustered, and that can cause a loss of functionality, destabilization of the membrane and a loss of its selective permeability [21] [22].

During cryopreservation, boar spermatozoa contains high content of polyunsaturated fatty acids and a low level of cholesterol in the plasma membrane, making them susceptible to peroxidation damage [23] [24]. In addition, free radicals produced by sperm are highly reactive groups of molecules, so they are very

susceptible to reacting with other molecules, oxidizing them, leading to a decrease in sperm motility, increased damage to sperm DNA, and decreased efficiency in sperm fusion in oocytes [25]. These modifications lead to a decrease in the fertilization rate and subsequent embryo development.

No consistency between researchers for *in vitro* fertilization rates with frozen-thawed epididymal semen or ejaculated semen. This study suggested that sperm affected the production of pig embryos *in vitro* efficiency, boar semen recovered from epididymides is less tolerant to freezing/thawing process than ejaculated spermatozoa. Sperm from ejaculate had production pig embryos *in vitro* better than sperm from the epididymis. Our result differs from those of Cunha *et al.* (2019) [26] and Rath and Niemann (1997) [27], but is similar to those of Matás *et al.* (2010) [28] and Rodriguez-Villamilet *et al.* (2016) [29]. According to Cunha *et al.* (2019) [26], there were no differences in the oocytes cleavaged, blastocyst formation rates between the epididymis and the ejaculate group. Meanwhile, Rath and Niemann (1997) [27] showed fertilization rates of epididymal semen higher than with ejaculated semen. Although Pamungkas *et al.* (2012) [19] indicated there were no significant differences in the normal fertilization rate between epididymal and ejaculated spermatozoa, the normal fertilization rate of the ejaculated group was higher than epididymal group (48.78% vs 42.98%, respectively). Even Pamungkas *et al.* (2012) [19] showed that the polyspermic fertilization rate of the epididymal group was higher than ejaculated group (18.42% vs 17.89%, respectively). Otherwise, Matás *et al.* (2010) [28] showed that epididymal spermatozoa have a lower response to capacitation treatments than their ejaculated counterparts. In conclusion, in Matás *et al.* (2010) [28], epididymal and ejaculated spermatozoa respond differently to *in vitro* capacitation treatments. Similarly, Rodrigues-Villamil *et al.* (2016) [29] showed a lower embryo rate in the epididymal group than those obtained in the ejaculate group.

Although Harkema *et al.* (2004) [30] and Okazaki *et al.* (2012) [31] suggested that the rate of epididymal spermatozoa reaches the oviduct *in vivo* lower than that of ejaculated spermatozoa and they show lower fertility, in **Table 4** of this study, it was found that pregnancy rate of ejaculated group was similar to that of epididymal group (60% vs 60%).

After removing the testicle, the sperm is kept in the tail of the testicle, and the sperm is exposed to a fluid at the time of ejaculation [26]. These fluids contain several substances that are an important influence on sperm viability and motility in the female reproductive tract, such as ions, lipids, energy substrates, organic compounds and proteins [32]. These substances are known to be important for the fertilization process. In addition, the sperm recovered by ejaculation have had contact with seminal plasma and seminal plasma contained factors are known to confer resistance to cold injuries during cryopreservation of boar spermatozoa [33]. The proteins in seminal plasma play an important role in membrane stability, heparin-binding, sperm capacitation and the formation of sperm-oocyte interaction [32]. Meanwhile, sperm recovered from the epididymis will not be exposed to protein in seminal plasma, such as sperm recovered

by ejaculation. This lack of exposure to components contained in seminal plasma can affect sperm capacitation and fertilizing potential of sperm recovered from the epididymis [26]. Moreover, the sperm of boars from the epididymal are very sensitive to the female genital tract *in vivo* and more vulnerable to uterine barriers [34].

In this study, although the production of pig embryos of sperm recovered from the epididymis of *Co Binh Thuan* pig was lower than that of sperm recovered by ejaculated cryopreservation of spermatozoa obtained from the epididymis and using them for artificial insemination or IVF embryo production is essential. Cryopreservation of spermatozoa recovered from the epididymis remains an effective method to preserve the genetic material of rare males who have died due to objective or subjective factors.

In **Table 5** of our study, although there was no difference in cleavage rate, blastocyst formation rate and the average number of cells/blastocysts, the hatching blastocyst rate was different between the breeds ($P > 0.05$). According to Namula *et al.* (2021) [35], the differences between the breed influence the fertility of frozen-thawed boar. Namula *et al.* (2021) [35] observed that there were differences in fertility of frozen-thawed boar spermatozoa among breeds, fertilization rate of spermatozoa from the micro- and mini-pig boar was lower than Large White boar. Waterhouse *et al.* (2006) [18] showed that there are differences in the composition of fatty acids in the sperm cell membrance among breeds that influence the fertility of frozen-thawed semen.

In conclusion, the ejaculated and epididymal sperm of native Vietnamese pigs were successfully frozen. We succeeded in creating embryos *in vitro* and pregnant pigs after artificial insemination from frozen-thawed semen in three native Vietnamese pig breeds for the first time. The use of the ejaculated sperm improved the production of native pig embryos *in vitro* efficiency.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Dang-Nguyen, T., TICH, N.K., Nguyen, B.X., Ozawa, M., Kikuchi, K., Manabe, N., Ratky, J. Kanai, Y. and Nagai, T. (2010) Introduction of Various Vietnamese Indigenous Pig Breeds and Their Conservation by Using Assisted Reproductive Techniques. *Journal of Reproduction and Development*, **56**, 31-35.

https://doi.org/10.1262/jrd.09-165K

[2] Nga, B. and Sarah, G. (2019) Vietnam African Swine Fever Update (Report). GAIN Report, Voluntary Report: VM2019-0067.

[3] Rodrigues-Martinez, H. and Wallgren, M. (2011) Advances in Boar Semen Cryopreservation. *Veterinary Medicine International*, **2011**, Article ID: 396181. https://doi.org/10.4061/2011/396181

[4] Yeste, M., Rodrígues-Gil, J.E. and Bonet, S. (2017) Artificial Insemination with Frozen-Thawed Boar Sperm. *Molecular Reproduction Development*, **84**, 802-813. https://doi.org/10.1002/mrd.22840

[5] Kikuchi, K., Kaneko, H., Nakai, M., Somfai, T., Kashiwazaki, N. and Nagai, T. (2016) Contribution of *in Vitro* System to Preservation and Utilization of Porcine Genetic Resources. *Theriogenology*, **86**, 170-175. https://doi.org/10.1016/j.theriogenology.2016.04.029

[6] Nguyen, B.X., Kikuchi, K., Uoc, N.T., Dang Nguyen, T.Q., Linh, N.V., Men, N.T. and Nagai, T. (2015) Production of Ban Miniature Pig Embryos by *in Vitro* Fertilization: A Comparative Study with Landrace. *Animal Science Journal*, **86**, 487-493. https://doi.org/10.1111/asj.12317

[7] Linh, N.V., Somfai, T., Nguyen, T., Nhung, N.T., Hong, N.T., Dat, N.T., Thinh, N.H., Khanh Van, N., Van Quyen, D., Chu, H.H., Son, N.T. and Kikuchi, K. (2018) Optimization of the *in Vitro* Fertilization Protocol for Froenepididymal Sperm with Low Fertilization Ability in Ban-A Native Vietnamese Pigs. *Animal Science Journal*, **89**, 1079-1084. https://doi.org/10.1111/asj.13045

[8] Nguyen, N.T., Bui, N.X., Nguyen, V.L., Van Khanh, N., Kikuchi, K., Nguyen, H.T., Nguyen, H.T., Nguyen, H.T., Van Dong, Q., Chu, H.H., Cuc, N.T.K. and Somfai, T. (2020) Optimization of *in Vitro* Embryo Production and Zygote Vitrification for the Indigenous Vietnamese Ban Pig: The Effect of Different *in Vitro* Oocytes Maturation Systems. *Animal Science Journal*, **91**, e13412. https://doi.org/10.1111/asj.13412

[9] The Government of Vietnam (2020) Decree No. 13/2020/ND-CP, Elaboration the Law on Animal Husbandry. https://thuvienphapluat.vn/

[10] SATREPS Project (2020) Manual for Database and Cryopreservation System for the Gene Banking. SATREPS/Vietnam Protocols 2020.

[11] Kikuchi, K., Nagai, T., Kashiwazaki, N., Ikeda, H., Noguchi, J., Shimada, A., Soloy, E. and Kaneko, H. (1998) Cryopreservation and Ensuing *in Vitro* Fertilization Ability of Boar Spermatozoa from Epididymides Stored at 4 Degrees C. *Theriogenology*, **50**, 615-623. https://doi.org/10.1016/S0093-691X(98)00166-6

[12] Yoshioka, K., Suzuki, C. and Onishi, A. (2008) Defined System for *in Vitro* Production of Porcine Embryos Using a Single Basic Medium. *Journal Reproduction Development*, **54**, 208-213. https://doi.org/10.1262/jrd.20001

[13] Van Khanh, N., *et al.* (2021) Optimization of Donor Cell Cysle Synchrony, Maturation Media and Embryo Culture System for Somatic Cell Nuclear Transfer in the Critically Endangered Vietnamese Ỉ Pig. *Theriogenology*, **166**, 21-28. https://doi.org/10.1016/j.theriogenology.2021.02.008

[14] Kikuchi, K., Onishi, A., Kashiwazaki, N., Iwamoto, M., Noguchi, J., Kaneko, H., Akita, T. and Nagai, T. (2002) Successful Piglet Production after Transfer of Blastocyst Produced by a Modified *in Vitro* System. *Biology of Reproduction*, **66**, 1033-1041. https://doi.org/10.1095/biolreprod66.4.1033

[15] Suzuki, K., Asano, A., Ericksson, B., Niwa, K., Nagai, T. and Rodriguez-Martinez, H. (2002) Capacitation Status and *in Vitro* Fertility of Boar Spermatozoa: Effects of Semi-

nal Plasma, Cumulus-Oocytes-Complexes-Conditioned Medium and Hyaluronan. *International Journal of Andrology*, **25**, 84-93. https://doi.org/10.1046/j.1365-2605.2002.00330.x

[16] Worwood, D. (2007) Artificial Insemination for Beginners: The Insemination Process. Utah State University, Logan.

[17] Watson, P.F. (2000) The Causes of Reduced Fertility with Cryopreserved Semen. *Animal Reproduction Science*, **60-61**, 481-492. https://doi.org/10.1016/S0378-4320(00)00099-3

[18] Waterhouse K.E., Hofmo, P.O., Tverdal, A. and Miller, R.R. (2006) Within and between Breed Differences in Freezing Tolerance and Plasma Membrance Fatty Acid Composition of Boar Sperm. *Reproduction*, **131**, 887-894. https://doi.org/10.1530/rep.1.01049

[19] Pamungkas, F.A., Setiadi, M.A. and Karja, N.W.K. (2012) Characteristics and *in Vitro* Fertilization Ability of Ram Spermatozoa: Comparison of Epididymal and Ejaculated Spermatozoa. *Media Peternakan*, **35**, 38-44. https://doi.org/10.5398/medpet.2012.35.1.38

[20] Leahy, T. and Gadella, B.M. (2011) Sperm Surface Changes and Physiological Consequences Induced by Sperm Handling and Storage. *Reproduction*, **142**, 759-778. https://doi.org/10.1530/REP-11-0310

[21] Vadnais, M.L. and Althouse, G.C. (2011) Characterization of Capacitation, Cryoinjury, and the Role of Seminal Plasma in Porcine Sperm. *Theriogenology*, **76**, 1508-1516. https://doi.org/10.1016/j.theriogenology.2011.06.021

[22] Yeste, M. (2016) Sperm Cryopreservation Update: Cryodamage, Markers and Factors Affecting the Sperm Freezability in Pigs. *Theriogenology*, **85**, 47-64. https://doi.org/10.1016/j.theriogenology.2015.09.047

[23] Esmaeili, V., Shahverdi, A.H., Moghadasian, M.H. and Alizadeh, A.R. (2015) Dietary Fatty Acids Affect Semen Quality: A Review. *Andrology*, **3**, 450-461. https://doi.org/10.1111/andr.12024

[24] Cerolini, S., Maldjian, A., Surai, P. and Noble, R. (2000) Viability, Susceptibility to Peroxidation and Fatty Acid Composition of Boar Semen during Liquid Storage. *Animal Reproduction Science*, **58**, 99-111. https://doi.org/10.1016/S0378-4320(99)00035-4

[25] Agarwal, A., Prabhakaran, S. and Sikka, S. (2007) Clinical Relevance of Oxidative Stress in Patient with Male Factor Infertility: Evidence-Based Analysis. *American Journal of Reproductive Immunology*, **59**, 2-11. https://doi.org/10.1111/j.1600-0897.2007.00559.x

[26] Cunha, A.T.M., Carvalhon, J.O., Guimarães, A.L.S., Leme, L.O., Caixeta, F.M., Viana, J.H.M. and Dode, M.A.N. (2019) Bovine Epididymal Spermatozoa Treatment for *in Vitro* Fertilization: Heparin Accelerates Fertilization and Enables a Reduction in Coincubation Time. *PLOS ONE*, **14**, e0209692. https://doi.org/10.1371/journal.pone.0209692

[27] Rath, D. and Niemann, H. (1997) *In Vitro* Fertilization of Porcine Oocytes with Fresh and Frozen-Thawed Ejaculated or Frozen-Thawed Epididymal Semen Obtained from Identical Boars. *Theriogenology*, **47**, 785-793. https://doi.org/10.1016/S0093-691X(97)00034-4

[28] Matás, C., Sansegundo, M., Ruiz, S., García-Vaquez, F.A., Gadea, J., Romar, R. and Coy, P. (2010) Sperm Treatment Affects Capacitation Parameters and Penetration Ability of Ejaculated and Epididymal Boar Apermatozoa. *Theriogenology*, **74**, 1327-1340. https://doi.org/10.1016/j.theriogenology.2010.06.002

[29] Rodrigues-Villamil, P., Hoyos, M.V., Maritns, J.A., Oliveira, A.N., Aguiar, L.H., Moreno, F.B., Velho, A.L.M.C.S., Monteiro-Moreira, A.C., Moreira, R.A., Vasconcelos, I.M., Bertolini, M. and Moura, A.A. (2016) Purification of Binder of Sperm Protein 1 (BSP1) and Its Effects on Bovine *in Vitro* Embryo Development after Fertilization with Ejaculated and Epididymal Sperm. *Theriogenology*, **85**, 540-554. https://doi.org/10.1016/j.theriogenology.2015.09.044

[30] Harkema, W., Visser, I., Soede, N.M., Kemp, B. and Woelders, H. (2004) Capacity of Boar Spermatozoa to Bind Zona Pellucida Proteins *in Vitro* in Relation to Fertilization Rates *in Vivo*. *Theriogenology*, **61**, 227-238. https://doi.org/10.1016/S0093-691X(03)00212-7

[31] Okazaki, T., Akiyoshi, T., Kan, M., Mori, M., Teshima, H. and Shimada, M. (2012) Artificail Insemination with Seminal Plasma Improves the Reproductive Performance of Frozen-Thawed Boar Epididymal Spermatozoa. *Journal of Andrology*, **33**, 990-998. https://doi.org/10.2164/jandrol.111.015115

[32] Juyena, N.S. and Stelletta, C. (2012) Serminal Plasma: An Essential Attribute to Spermatozoa. *Journal of Andrology*, **33**, 536-551. https://doi.org/10.2164/jandrol.110.012583

[33] Okazaki, T. and Shimada, M. (2012) New Strategies of Boar Sperm Cryopreservation: Development of Novel Freezing and Thawing Methods with a Focus on the Roles of Seminal Plasma. *Animal Science Journal*, **82**, 623-629. https://doi.org/10.1111/j.1740-0929.2012.01034.x

[34] Cristina, S.U., Karen, A.L., Francisco, A.G.V., Jon, R.A. and Carmen. M. (2021) Epididymal and Ejaculated Sperm Functionality Is Regulated Differently by Periovualatoryoviductal Fluid in Pigs. *Andrology*, **9**, 426-439. https://doi.org/10.1111/andr.12902

[35] Namula, Z, Isumi, Y., Sato, Y., QuynhAnh Le, Lin, Q., Takebayashi, K., Hirata, M., Tanihara, F., Thongkitidilok, C. and Otoi, T. (2021) Improvement of the *in Vitro* Fertilization and Embryo Development Using Frozen-Thawed Spermatozoa of Microminipigs. *Archives Animal Breeding*, **64**, 265-271. https://doi.org/10.5194/aab-64-265-2021