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COLLECTION, FREEZING OF BAN EPIDIDYMAL SPERM AND EVALUATION OF *IN VITRO* FERTILITY AFTER THAWING

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SUMMARY

Many researches on characteristics of porcine ejaculated semen on popular commercialized breeds collecting at breeding centers or households with pig breeding services have been carried out in Vietnam. However, very few investigation had been dealt with epididymal sperms, especially the ones from boar of Ban, a popular Vietnam native mini-pig breed which is usually freely raised in far rural and mountainous regions in some provinces of Northern Vietnam. In the present study, we surveyed on criteria of epididymal sperms from 4 Ban boars at collection, after freezing and thawing processes, and tested their fertility by an *in vitro* fertilization and embryo culture experiment. Approximate volume of sperm collected from a Ban boar did not differ (3-4 mL), with sperm concentration from 6.4 x 10^9 to 11.3 x 10^9 sperm/mL. Motility varied from 8.7 to 27.0%, whereas vitality was from 58.0 to 85.6%. After freezing and thawing, the motility of sperm slightly decreased to values of 6.3 to 25.7%, and viability significantly decreased to values of 41.3 to 79.6%. No difference was found between rates of abnormal morphology before and after freezing and thawing (10.6 to 31.0% and 12.0 to 32.0%, respectively). A test by *in vitro* fertilization with Landrace oocytes revealed that two sperm lots had acceptable *in vitro* fertility with rates of blastocyst formation from 14.4 to 18.8%. In conclusion, a study on collection, freezing and fertility testing of epididymal sperm collected from Ban boars has been carried out. The results of the present study could contribute necessary information as well as standardized sperm lots as important materials to further research on *in vitro* fertilization of Ban in Vietnam.

Keywords: *Ban pig, epididymis, sperm collection, in vitro fertilization*

INTRODUCTION

Pig, with similarities in physiology, is a good research models for human bio-mechanisms and source of organs for xeno-transplantation, especially mini-pig with compatible organ size to human. Vietnam has a large diversity of native pig breeds, many of those are mini-pigs, with precious features in food and bio-medical aspects, including low/no PERV insertion in their genome (Ishihara *et al*., 2018). However, many Vietnam native pig breeds are now facing high decrease and/or extinction due to their disadvantages in economic productivity (Dang-Nguyen *et al*., 2010). Conservation of Vietnam native pig breeds is therefore crucial. Among them, Ban, a miniature pig which is rather popular raised with adequate population in far rural and mountainous Northern Vietnam regions, is an ideal subject and model for research and development of assisted reproductive techniques towards conservation, including sperm cryopreservation. Manual ejaculate

collection of semen through gloved-hand method is popular for commercial pig breeds such as Landrace or Great White. However, this method could hardly apply for Ban pigs, due to their free raising behaviors. Therefore, epididymal sperm collection from slaughtered Ban boars is more practical, and more essential as being a model for endangered or rare pig breeds whose individuals for conservation are commonly available only when slaughtered, or mostly dead.

There are few studies on cryopreservation and utilization of epididymal sperms. Successful epididymal sperm freezing has been successful only in some species: cat (Goodrowe and Hay, 1993), cattle (Amann and Griel, 1974) and pig (Nagai *et al*., 1988). Epididymal and ejaculated semen show different characteristics including fertility (Foote, 2000), cooling (Hammond, 1930; Walton, 1930) and freezing (Songsasen and Leibo, 1998). Spermatozoa collected from epididymis has higher resistance to cold shock than ejaculated ones (Losley and Bogart, 1944), therefore supporting successful *in vitro* fertilization then suggesting that they can be more suitable for cryopreservation (Jiang *et al*., 1991; Rath and Niemann, 1997). Sperms from epididymis and ejaculated sperms are different in aspects of morphological (loss of cytoplasmic droplets) (Hewitt *et al*., 2001) and functional (membrane changes related to resistance to osmotic stress) (Watson, 2000). Despite of low quantity and need of sperm activation before fertilization, epididymal sperms could maintain their viability as well as fertility when being kept at 4°C after 1-2 days after collection (Kikuchi *et al*., 1998), making it a promising method to conserve male germ cells, especially in individuals whose breed or species is rare or endangered. The present study was carried out to survey on collection of Ban epididymal sperms with criteria, freezing and thawing efficiency, and testing their *in vitro* fertility in an established *in vitro* embryo production system.

MATERIALS AND METHODS

Epididymal sperm collection, freezing and thawing

Epididymal sperm collection and freezing were carried out according to the method by Kikuchi *et al.* (1998) with minor modification (Hiep *et al.*, 2014). Briefly, whole testes of 4 Ban boars of 12-14 months old were detached and transferred to laboratory right after slaughtering in physiological saline (NaCl 0.9% supplemented with antibiotics). After testes being washed many times by PBS, epididymis were carefully isolated from testes, luminal fluid containing spermatozoa was extruded from the distal portion of the cauda epididymis by air pressure from a syringe. Total volume of luminal fluid was recorded. A small volume of the fluid was used for post-collection evaluation of concentration, motility, viability and abnormality (Figure 1). Criteria of sperms (motility, viability and abnormality) were evaluated as described previously (Hiep *et al*., 2014). The rest of the fluid was diluted with 30 mL of collecting solution rewarmed to room temperature. Sperm suspension in a 50-mL glass centrifugation tube was placed in water (2000 mL at room temperature) and transferred to a refrigerator to be cooled down to 15°C in 3 hours. Subsequently, the fluid was centrifuged at 1200×g for 10 minutes and the supernatant discarded. Precipitated spermatozoa were gently resuspended in 5 mL NSF-I extender. It took about 1 hour for centrifugation and resuspension at 15°C. Resuspended spermatozoa were cooled to 4°C within 2 hours. Spermatozoa resuspended in NSF-I were then mixed with an equal volume of NSF-II, which determined the final 3% (v/v) concentration of glycerol as cryoprotectant. The sperm concentration was adjusted to 1×10^8 cells/mL. The sperm suspension was transferred to 0.25-mL plastic straws, which were placed in liquid nitrogen vapor for 10 min and finally stored in liquid nitrogen.

Thawing of sperm was carried out as previously described (Hiep *et al.*, 2014). Straws

were taken out of liquid nitrogen container and put immediately into glass of water at 37°C, then sperms were freed from the straws into a tube with TCM-199 medium to be washed and centrifuged. Supernatant was eliminated, and pellet was resuspended in about 200 µL of TCM-199 medium with Earle's salts, pH adjusted to 7.8. It was then transferred into in a 30-mm petri dish, covered by paraffin oil and activated at 37ºC for 15 minutes in an incubator. Sperms were then used for either evaluation of post-thawing criteria: motility, viability, and abnormality, or *in vitro* fertilization.

In vitro fertility evaluation

Oocyte collection, *in vitro* maturation, *in vitro* fertilization and embryo culture were carried out as previously described (Kikuchi *et al.*, 2002, Hiep *et al.* 2014). Briefly, ovaries of Landrace sows were collected from slaughter houses, rinsed and transported to the laboratory in phosphate buffer saline (PBS) solution at 35˚C in less than 3 hours. The ovaries were washed several times in PBS and cumulus oocytes complexes (COCs) were aspirated from ovarian follicles using a scalpel blade. COCs were selected in TCM-199 medium with Hanks' salts supplemented with 5% FBS, 20 mM HEPES, 100 IU/mL penicillin G potassium and 0.1 mg/mL streptomycin sulfate under a stereo microscope. *In vitro* maturation was carried out in groups of less than 50 oocytes in a well of 4-well disks containing 500 µL of maturation media supplemented with 0.6 mM cysteine, 50 µM β-mercaptoethanol (β-ME), 10µg/mL FSH and LH, and 1.0 mmol/L dibutyryl cyclic adenosine monophosphate (dbcAMP) at 39ºC, 5% CO_2, 5% O_2, 90% N_2 in humidified air. After 20-22 hours, COCs in groups were then transferred into other disks containing the same medium but without hormones and dbcAMP for a further culture of 22-24 hours. A suitable volume of sperms was diluted in Pig-FM medium after determining the concentration of sperm. 10 µL of the correspondent sperm dilution was introduced into the 90-µL IVF droplets containing the oocytes to a final concentration of 10^6 sperm/mL and co-incubated at 39ºC 38.5ºC under 5% CO_2 for 3 hours. At the end of IVF, spermatozoa and cumulus cells were removed from the surface of the zona pellucida by gentle pipetting with a fine glass pipette. Then, oocytes were cultured for 500-µL PZM3 medium for 6 days (Nhung *et al.*, 2019) in 4-well dishes in an atmosphere of 5% CO_2, in humidified air at 38.5ºC. At day 2 and day 7 of *in vitro* culture (day of fertilization is defined as day 0), embryos were recorded for ones which could reach to cleavage and blastocyst stage, as well as hatching blastocyst number.

Statistical analysis

Data were expressed as number and percentages were under the form of mean ± SEM values. Data were analyzed by Student's t-test or one-way ANOVA on MS Excel Software.

RESULTS AND DISSCUSSION

Epididymal sperm criteria after collection

Results on criteria evaluating after collection of sperm from Ban epididymis are shown in Table 1, surveying on collected luminal fluid volume, concentration, motility, viability and abnormality.

The volumes of collected luminal fluid were from 3 to 4 mL, not much variable between boars. Sperm concentration, motility, viability, and abnormality, on the other hand, varied significantly between boars, in which TLB 001 and TLB 004 had lowest motility and viability (8.7% and 58%, respectively), and TLB 003 had highest volume, concentration, motility and viability in compared to the others. Motility of sperms after collection in the present study is lower than those from Landrace in the previous studies (Kikuchi *et al*., 1998). The volumes of luminal fluid collected from epididymis of this study are in accordance to those in Nguyen *et al.* (2015), which is 3.8 mL/boar. It is lower than that of Landrace counterparts which is much higher (13.0 mL/boar, Nguyen *et al.*, 2015). However, remarkably, the concentration of sperm collected from Ban epididymis in the

present study is much higher than those from Ban and even Landrace counterparts in Nguyen *et al.*, (2015) (6406-11337 million/mL vs. 1240 and 4160 million/mL, respectively, about 1.5 to 9 folds), whereas the other criteria except abnormal rate are not largely different. This might because the age of boars using in the present study was much higher than that of boars in Nguyen *et al.* (12-14 months old vs. 4 months old, respectively). Similar to some other Vietnamese native pigs, Ban has remarkable earlier age of maturity which is commonly about 3 months old, and boars could fertilize female and produce piglets (Dang-Nguyen, 2010). However, it might be inferred from the present study that maturity of Ban boars still significantly grow after 3 months old revealing a higher amount of sperm available in epididymis. Further study should be conducted to address this issue.

Table 1. Evaluation of Ban epididymal sperms after collection.

No	Boar code	Collection volume (mL)	Motility (%)	Sperm concentration (million/mL)	Viability (%)	Abnormality (%)
1	TLB 001	3.2	13.0 ±1.1	6406 ± 137	58.0 ± 1.5	31.0 ± 0.5
2	TLB 002	3	20.0 ± 1.4	9332 ± 119	76.3 ± 0.8	12.3 ± 0.6
3	TLB 003	4	27.0 ± 2.0	11337 ± 56	85.6 ± 0.6	10.6 ± 1.4
4	TLB 004	3.5	8.7 ± 3.6	8772 ± 15	73.3 ± 0.6	11.0 ± 1.7

TLB: “Tinh lợn Bản”, abriviation for Vietnamese “Ban pig sperm”. Percentages are shown as mean ± SEM.

Viability of sperm lots TLB 002, TLB 003 and TLB 004 were 73.3, 76.3 and 86.5, respectively, higher than national standard for artificial insemination in native pigs (>70%, Ministry of Agriculture and Rural Development, Vietnam, 2013). Motility is much lower than than national standard (Ministry of Agriculture and Rural Development, Vietnam, 2013) due to the fact that epididymal sperm is not yet ready for normal insermination as in natural mating or collection of ejaculated sperm. Sperm concentrations in the present study are from 6406 to 11337 million/mL, which is much higher and could be diluted for artificial insemination which needs only a concentration of >200 million/mL. Nevertheless, sperms in luminal fluid with extremely high concentration are not activated i.e. not ready to fertilize. Only a further step of sperm activation which require TCM-199 with pH 7.8 and incubation at 37ºC could ensure fertility of epididymal sperms, which also limit its applicability in artificial insemination. Therefore, epididymal sperm collection should serve only conservation and *in vitro* embryo production purposes.

In the present study, we did not monitor changes in sperm criteria during freezing process like Kikuchi *et al.* (1998). Efficiency was only evaluated after freezing and thawing by criteria of motility, viability and abnormality, as shown in Table 2.

Epididymal sperms were proven to be effectively used for *in vitro* embryo production by *in vitro* fertilization (Kikuchi *et al.*, 1998), however, commonly after freezing-thawing process, motility and viability decreased, and abnormality increased (Kikuchi *et al.*, 1998; Nguyen *et al.*, 2015). Researches in other species also showed a decrease in sperm quality and subsequent fertility after freezing (Bailey *et al.*, 2000; Watson, 2000). Pig has more damage of sperm after freezing than other species (Rath *et al.*, 2009). This might because of fragility of porcine sperm membrane (Johnson *et al.*, 2000; Techakumphu *et al.*, 2013), and immediate change of temperature during thawing process relating to elimination, transportation or

replacement of proteins and lipid, leading to effects on cellular permeability and functions (Leahy and Gadella, 2011). In the present study, all sperm lots had decreased rates of viability, which varied from boar to boar. Boar quality and treatment of epididymis during freezing might be the reason of this variation (Pelaez *et al.*, 2006; Gil *et al.*, 2008). The results after freezing were in accordance with previous studies (Kikuchi *et al.*, 1998; Nguyen *et al.*, 2015) in aspects of viability and motility, however, no significant change in abnormal rates before and after freezing.

Fertility of sperms, especially frozen sperms, is not always reflected by after thawing sperm criteria, such as motility and viability. Therefore, despite of good results achieved in the previous evaluation of frozen-thawed sperms, we continued *in vitro* fertility experiment to test the fertility of Ban sperm by *in vitro* embryo productivity. Results of this experiment were shown in Table 3.

Table 2. Evaluation of Ban epididymal sperms after freezing-thawing.

No	Boar code	Motility (%)	Viability (%)	Abnormality (%)
1	TLB 001	10.2 ± 2.6	41.3 ± 2.0**	32 ± 1.1
2	TLB 002	16.6 ± 0.8*	70.6 ± 2.3*	13 ± 0.5
3	TLB 003	25.7 ± 1.4	79.6 ± 0.6*	12 ± 1.5
4	TLB 004	6.3 ± 1.1	63.3 ± 1.2**	12.6 ± 0.6

5 straws of each sperm lot were thawed for evaluation. Percentages are shown as mean ± SEM. Superscripts of *, ** and *** marked significantly difference between data after freezing-thawing and counterparts right after collection, with P<0.05, P<0.01, and P<0.001, respectively (see also Table 1).

Table 3. Evaluation of *in vitro* fertility of frozen Ban epididymal sperms.

No	Boar code	Total number of oocytes	Number (%) of oocytes with cleavage	Number (%) of blastocyst	Number (%) of hatching blastocyst (%)
1	TLB 001	90	31 (34.4 ± 2.9)^{c}	5 (5.5 ± 4.0)^{c}	0 (0.0 ± 0.0)^{c}
2	TLB 002	90	43 (47.7 ± 2.9)^{b}	13 (14.4 ± 2.2)^{b}	1 (1.1 ± 1.1)^{b}
3	TLB 003	90	49 (54.4 ± 4.4)^{a}	17 (18.8 ± 1.1)^{a}	4 (4.4 ± 1.1)^{a}
4	TLB 004	90	39 (43.3 ± 1.9)^{b}	7 (7.7 ± 2.9)^{c}	0 (0.0 ± 0.0)^{c}

3 replications were carried out. Percentages are shown as mean ± SEM. Superscripts in the same column reflect significant difference (P<0.05).

The results showed that sperm quality criteria after freezing-thawing were well-reflected in *in vitro* fertility evaluation test. Rates of cleavage and blastocyst formation were both higher in TLB 003 and TLB 002 lots than those of TLB 001 and TLB 004 lots. Only blastocyst from TLB 003 and TLB 002 lots could reach to hatching stage, which clearly indicate a strong viability and development of the embryos. The blastocyst rate in TLB 003 is higher than that in Nguyen *et al.*, 2015 (18.8% and 10.5%, respectively), and slightly higher than that of Landrace counterparts in the two other studies of the same system (Nguyen *et al.*, 1015: 15.3%; Hiep *et al.*, 2014: 16.7%). This might be because of the use of PZM3 as *in vitro* embryo culture medium, which has been shown to better support *in vitro* development of porcine embryos (Nhung *et al.*, 2019). All four lots of sperms could contribute to *in vitro*

fertilization with various achievement of blastocyst, however, only TLB 003 and TLB 002 meets the standards of *in vitro* embryo production, suitable for further researches.

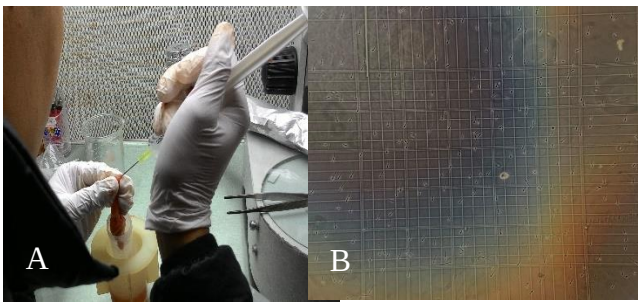

Figure 1. Collection and evaluation of Ban epididymal sperm: (A) Collection of luminal fluid by air pressure from a syringe; (B) Evaluation of sperm quality on phase-contrast microscope with a cell counting chamber.

CONCLUSION

Quantity and quality criteria of sperm collected Ban boar epididymis were evaluated, as well as those after freezing and thawing. The sperm lots were also tested for *in vitro* fertility. Two out of four sperm lots satisfied all criteria of sperm for *in vitro* production of Ban pig embryos. The results showed the possibility of effective collection and conservation of Ban boar epididymal sperm as a model of other Vietnamese native pig breeds, contribute to efforts in establishment of reproductive biotechnology system for effective conservation of native pig biodiversity in Vietnam.

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THU NHẬN, ĐÔNG LẠNH TINH TRÙNG LỢN BẢN TỪ MÀO TINH HOÀN VÀ ĐÁNH GIÁ KHẢ NĂNG THỤ TINH *IN VITRO* SAU GIẢI ĐÔNG

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TÓM TẮT

Nhiều nghiên cứu trên đối tượng tinh trùng thu bằng phương pháp xuất tinh thủ công trên các giống lợn thương phẩm phổ biến tại các trung tâm giống hoặc nông hộ đã được thực hiện ở Việt Nam. Tuy nhiên, có rất ít nghiên cứu trên đối tượng tinh trùng thu trực tiếp từ mào tinh hoàn, đặc biệt trên lợn Bản, một giống lợn mini bản địa thường được nuôi ở một số tỉnh miền núi vùng sâu, vùng xa phía Bắc Việt Nam. Trong nghiên cứu này, chúng tôi khảo sát các chỉ tiêu của tinh lợn thu từ mào tinh hoàn của 4 lợn Bản đực tại các thời điểm khi thu mẫu, sau khi đông lạnh và giải đông, cũng như kiểm tra khả năng thụ tinh bằng thí nghiệm thụ tinh ống nghiệm và nuôi phôi. Thể tích tinh trùng thu được từ một lợn Bản không quá khác biệt (3-4 mL), với nồng độ tinh trùng cao dao động từ 6,4 tới 11,3 tỷ tinh trùng/mL. Hoạt lực của tinh trùng tại thời điểm thu có giá trị từ 8,7 tới 27,0%, tỷ lệ sống đạt 58,0 tới 85,6%. Sau quá trình đông lạnh và giải đông, hoạt lực của tinh trùng giảm nhẹ xuống còn từ 6,3 tới 25,7%, và sức sống giảm đáng kể xuống chỉ còn 41,3 tới 79,6%. Tỷ lệ kỳ hình không khác biệt giữa trước và sau đông lạnh - giải đông. Việc kiểm tra thụ tinh ống nghiệm được tiến hành với trứng lợn Landrace cho thấy hai lô tinh trùng có khả năng thụ tinh đạt yêu cầu với tỷ lệ hình thành phôi nang từ 14,4 tới 18,8%. Tóm lại, nghiên cứu thu nhận, đông lạnh và kiểm tra khả năng thụ tinh đã được tiến hành với tinh trùng thu từ mào tinh hoàn lợn Bản. Kết quả thu được đã cung cấp các thông tin cần thiết cũng như các lô tinh trùng đạt tiêu chuẩn làm nguồn nguyên liệu quan trọng cho các nghiên cứu thụ tinh ống nghiệm lợn Bản tại Việt Nam.

Từ khóa: *Lợn Bản, mào tinh hoàn, thu nhận tinh trùng, thụ tinh ống nghiệm*