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Evaluation of the Congjiang Xiang Pig's Sperm Quality by Flow Cytometry

Yong Huang[1,2,3], Ting Gong[1,2,3], Li-Jie Meng[1,2,3], Gang Wu[3], Wei-Yong Wang[1,2,3], Hou-Qiang Xu[1,2,3], Xiang Chen[1,2,3], Yi Gao[1,2,3], Xian-Zhou Feng[1,2,3]

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ABSTRACT

Background: Sperm quality plays an important role in the animal industry. The study of the sperm quality of Congjiang Xiang pigs is limited and the Rh123/PI double staining has not been used to detect the sperm quality of boars. Thus, the study aimed to evaluate the sperm quality of the Congjiang Xiang pigs and the feasibility of the flow cytometry (FCM) method in assessing sperm quality in pigs.

Methods: Semen samples were evaluated for routine indicators of semen quality and using FCM with single staining and double staining to analyze the integrity of membrane and acrosome, the mitochondrial function. The sperm quality of the Congjiang Xiang pigs was compared with the Large White pigs.

Result: The semen of the Congjiang Xiang pigs had a lower sperm concentration than that of the Large White pigs (P<0.01). There were no significant differences in sperm vitality, sperm motility and abnormality between the two breeds (P>0.05). Single sperm staining revealed a lower sperm acrosomal integrity in the semen of the Congjiang Xiang pigs than that of the Large White pigs (P<0.01). No significant difference between the two breeds in membrane integrity and mitochondrial function was observed (P>0.05). Rh123/PI double staining demonstrated that the rate of live sperm with a normal mitochondrial function of the Congjiang Xiang pigs was significantly higher than that of the Large White pigs (P<0.05). The rate of necrotic sperms in the semen of the Congjiang Xiang pigs was significantly lower than that in the semen of the Large White pigs (P<0.05). Correlation analysis revealed a positive association between the rates of live sperm in the semen of the congjiang xiang pigs and their sperm motility (P=0.051). Our results suggest that Rh123/PI double staining with flow cytometry can provide more reliable information to assess the boar sperm quality.

Key words: Congjiang xiang pigs, Flow cytometry, Sperm quality.

INTRODUCTION

Using high-quality male gamete is vital for ensuring the production of valuable offspring, which is economical in the animal industry, especially in livestock breeding and aquaculture. Sperm quality is one of the best discriminators for fertilization potential (Hadi *et al*., 2021; Aksoy *et al*., 2012). In many new technologies, semen quality is the most important criterion to measure the suitability of the method. In the study of developing cryopreservation methods/ procedures (Li *et al*., 2021) and freezing extenders (Guo *et al*., 2021), freezing methods and so on, the sperm quality after thawing determines whether the formula and method are available. Semen analysis is an integral part of the work up for studying the breeding ability of different species, such as Holstein Friesian crossbred breeding bulls (Nag *et al*., 2021), Saanen goats (Lopes *et al*., 2021) and Coho salmon (Sandoval *et al*., 2021).

Conventional semen analysis recommended by the World Health Organization (WHO) includes the measurements of sperm morphology, motility, vitality and concentration, which are the main indexes to evaluate the properties of male fertility. This analysis is time/labor-consuming and a subjective scoring method. Although WHO had been proposed the guidelines for standardizing the procedure, the huge distinction of the result obtained by different technician or laboratory have been reported (Franken, 2015). The conventional semen analysis result only can explain a fraction of the total variation in fertility and other semen characteristics are essential to explain the remaining variation. Flow cytometry (FCM) can reduce the disadvantage of conventional sperm evaluation and it has been used to analyzing attributes of sperm, such as sex-sorted (Holden *et al*., 2020) and assessment of chilling injury

[1]Key Laboratory of Animal Genetics, Breeding and Reproduction in the Plateau Mountainous Region, Ministry of Education, Guizhou University, Guiyang 550025, Guizhou Province, China.
[2]Guizhou Provincial Key Laboratory of Animal Genetics, Breeding and Reproduction, Guiyang 550025, Guizhou Province, China.
[3]College of Animal Science, Guizhou University, Guiyang, 550025, Guizhou Province, China.

Corresponding Author: Ting Gong, Key Laboratory of Animal Genetics, Breeding and Reproduction in the Plateau Mountainous Region, Ministry of Education, Guizhou University, Guiyang 550025, Guizhou Province, China. Email: tgong@gzu.edu.cn

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in hypothermic stored boar spermatozoa (Jäkel *et al*., 2021). Broekhuijse *et al*. (2012) used FCM to analyze the mitochondrial membrane potential, the integrity of plasma membrane and acrosome of boar spermatozoa under different feeding environments because this technique can provide statistically reliable data, after analyzing several thousand cells at a single-cell level within a few minutes. The spermatozoa which can fertilize an oocyte *in vivo* must have intact plasma membranes, acrosomes and sufficient cell metabolism. Propidium Iodide (PI) is a dead/moribund sperm-specific fluorescent dye that can distinguish dead or alive sperm. Mitochondria provide the energy required for sperm motility (Rebelo *et al*., 2021; Freitas *et al*., 2017). Rh123 was the most widely used mitochondrial-specific probe, which could identify the sperm with an abnormal mitochondrial membrane potential (Vašíček *et al*., 2022). The integrity of the mammalian sperm plasma membrane and acrosome is important for the fertilizing rate. The spermatozoon must retain a normal acrosome so that the acrosome reaction can occur to facilitate fertilization. Fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA) can combine with the damaged acrosome and fluoresces green.

The Congjiang Xiang pig is known for its early sexual maturity, mini size. It also has the physiology and other traits which was similarity to humans (Meng *et al*., 2020). Although they display promising potential as an experimental model and top-grade pork source, there are limited studies on the reproductive profile of the boars, particularly on sperm quality. Increasing litter size is a goal of pig breeders and producers in many countries, semen quality is the key factor in determining the fertilization rate and the litter size of sows (Meng *et al*., 2020; Gong *et al*., 2021) and few studies have described the FCM method for evaluating semen quality in pigs. Therefore, in this study, FCM was applied to detect the integrity of plasma membrane, acrosomal and mitochondrial function of adult Congjiang Xiang pigs.

MATERIALS AND METHODS

Semen specimens collection and classification

The experiment period was from 2022 February to November. Three boars per breed of Congjiang Xiang pigs and Large White pigs were used in this study. A total number of 18 ejaculates (3 ejaculates per boar) were collected from six boars (aged 3 years) and sexually active at sampling, the same trained technician used the hand-glove method once a week. The spermatozoa-rich fraction was retained in a sterile thermal bottle and transported back to Guizhou provincial key laboratory of animal genetics, breeding and reproduction at Guizhou University at 37°C. The Guiyang Lvshengyuan Animal Husbandry Technology Development Co., Ltd. (SCXK: 81 20160007, SYXK (Qian) 2018-0010, Guiyang, China) provided the semen samples of the Congjiang Xiang pigs and the Large White pigs. The seminal fluid was lique fied for 30 min at 37°C.

Semen analysis

Each sample was divided in to two parts. One part was used for routine detection and the other part was used for FCM. Preliminary semen characteristics (color, smell, state and pH) of the samples were obtained. Per sample would be recorded and calculated the concentration, vitality, motility and abnormality under the microscope (Hadi and Ali, 2021). All samples were evaluated by the sophisticated observers.

Single staining and FCM examination

Integrity of plasma membrane

Before staining, the spermatozoa was washed and adjusted to 5×10^6 sperms/mL by DPBS. Each sample (1 mL) was incubated with 5 µL PI dye solutions (1 mg/mL) for 15 min in the dark at 37°C. For eliminating the excess dye, the spermatozoa must wash by DPBS and then centrifuged. In FCM analysis, a flow rate of 2000 cells/s was used to analyze 10,000 sperms in each sample by flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA). The application of forward scatter (FSC) and side scatter (SSC) was amplified and the FSC was obtained at 400 mW power output. The SSC was obtained at 365 mW power output. The logarithmic amplification was performed for fluorescence channel Fluorescence detector 2 (FL2).

Sperm mitochondrial function

RH123 (Beyotime, Shanghai, China) was diluted to 1 mg/mL in dimethyl sulfoxide (DMSO: Solarbio, Beijing, China). 2 µL Rh123 dye solution were added to 1 mL sample at 37°C in the dark for 15 min. In FCM analysis, the flow rate and the power of FSC and SSC were set as described in PI staining. The logarithmic amplification was performed for fluorescence channel Fluorescence detector 1 (FL1).

Acrosome integrity

FITC-PNA (Thermo Fisher Scientific, Waltham, MA, USA) dyes were adjusted to 1 mg/mL by DMSO. 1 mL sperm sample was stained with 2 µL FITC-PNA dye solution in the dark 15 min and washed with DPBS to remove background staining (Zhu *et al*., 2019). A total of 10,000 sperms were analyzed using FCM with fluorescence channel FL1.

Rh123/PI double staining and FCM examination

In Rh123/PI double staining, 1 mL sample was stained with 10 µL RH123 (1 µg/µL) for 15 min, at 37°C in the dark and mixed with 10 µL PI (1 µg/µL) for 5 min at the same condition. After the double staining, the sample was washed twice times and resuspended by 1 mL DPBS. The sample was analyzed by FCM immediately. Fluorescent measurements were compensated to minimize spillover fluorescence between the red and green spectra. The compensation of green fluorescent was 24 and the compensation of red fluorescent was 0.

Fluorescence microscope observation

After staining, 10 µL of the sperm suspension was placed on a clean slide to detect the sperm fluorescence staining

by using the Nikon ECLIPSE-Ni+DS-Ri2 fluorescence microscope (Nikon Instruments Inc., Tokyo, Japan) with NIS-Elements BR acquisition and analysis software version 5.01 (Nikon Instruments Inc., Tokyo, Japan).

Statistical analysis

All data were expressed as mean±standard deviation (mean±SD). One-way analysis of variance (ANOVA) was used to determine the results of conventional semen parameters and FCM. All statistical data analys was performed with SPSS 13.0 software (IBM Corporation, Armonk, NY, USA) and $P<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Sperm function assessment in a semen sample is essential to analyze potential animal fertility. Laboratory semen features are correlated to farm animals' farrowing rates and litter sizes (Singh *et al*., 2019; Mcpherson *et al*., 2014). According to previous studies, the result of routine semen evaluation is subjective and insensitive to monitor the testicular function because routine semen evaluation only can detecte the samples with inferior quality (Andrade *et al*., 2010). FCM has been developed, which is recognized as a valuable method for assessing sperm quality (acrosomal integrity, mitochondrial function and sperm plasma membrane integrity) in reproductive toxicology, veterinary science and clinical andrology. The Congjiang Xiang pig is one kind of Chinese minipig breed with high economic value (Meng *et al*., 2020; Gong *et al*., 2021). However, their specific male reproductive characteristics were less studied. The present study provides useful information on the sperm function of Congjiang Xiang pigs for their potential fertility *via* the conventional method and FCM evaluation.

Routine detection of semen quality

Both semen of Congjiang Xiang pigs and Large White pigs smelled slightly fishy. The state of semen was not different between the two breeds, but the color of the Congjiang Xiang pigs was milky white and that of the Large White pigs were light gray. The pH of the Large White pigs (pH=7.3) was lower than the Congjiang Xiang pigs (pH=7.5). There was no significant difference in sperm vitality, motility and abnormality ($P>0.05$, data not shown). The sperm deformation rate of Congjiang Xiang pigs (8.83±2.50) was expected and acceptable in healthy boars (Wolf, 2009).

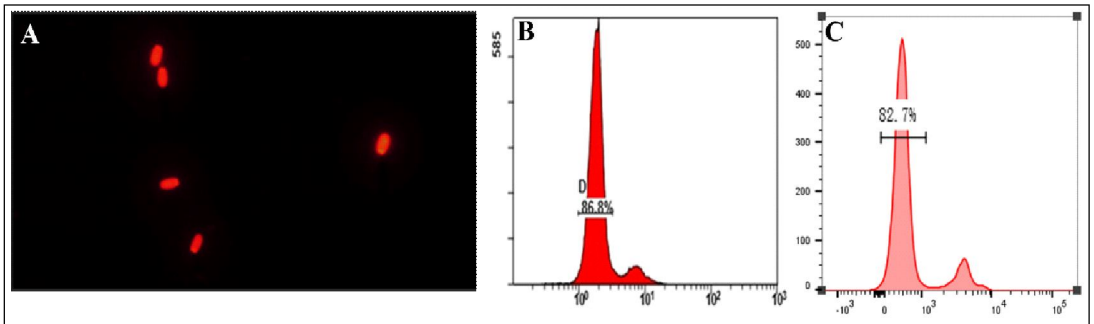
The differences in boar fertility are mainly due to genetics (Wolf, 2009). Smital and Lopez analyzed the semen quality of different breeds using the semen routine analysis method and the sperm concentration of different breeds was significantly different (Lopez Rodriguez *et al*., 2017). Compared with the Congjiang Xiang pigs, the sperm concentration of the Large White pigs was significantly higher (101.25±4.32 vs 70.38±5.09×10^6 sperm/mL, $P<0.01$). Different sperm concentrations and sperm numbers would result in different litter sizes of boar (Kommisrud *et al*., 2002; Johnson *et al*., 2000). Sperm concentration plays an important role in artificial insemination and the fertilization process because artificial insemination centers tend to dilute the ejaculates to introduce superior genes. From the sperm concentration perspective, the Congjiang Xiang pig is likely to have a weaker reproductive ability than the Large White pig.

Sperm membrane integrity

The integrity of the sperm membrane is one of the most important indexes in assessing sperm quality for predicting male fertility (Wysokińska *et al*., 2021). As shown in Fig 1, the sperm membrane integrity of the Congjiang Xiang pigs was 85.01±1.7%, which showed no substantial difference from that of the Large White pigs (80.81±1.93%, $P>0.05$).

Sperm mitochondrial activity

As shown in Fig 2, the sperm mitochondrial activity of the Congjiang Xiang pigs was 75.17±2.38% (Fig 2B) and the sperm mitochondrial activity of the Large White pigs was 70.76±1.89% (Fig 2C). Mitochondria play a decisive factor in providing the energy required for sperm motility (Freitas *et al*., 2017). They are very important to the whole process



A: Red fluorescence was centralized on the membrane-damaged sperm head under the fluorescence microscope (× 400); B-C: The sperm plasma membrane integrity in sperm of Congjiang Xiang pigs (B) and Large White pigs (C). PI^+ shows sperms with the damaged plasma membrane and PI^- sperms show sperm with the intact plasma membrane. The plasma membrane integrity is defined as either alive or dead.

Fig 1: Sperm PI fluorescent staining and FCM result.

of gamete production and reproduction, such as affecting reactive oxygen species (ROS) level (Murphy, 2016). However, there were no significant differences between Congjiang Xiang pigs and Large White pigs in this study (P>0.05).

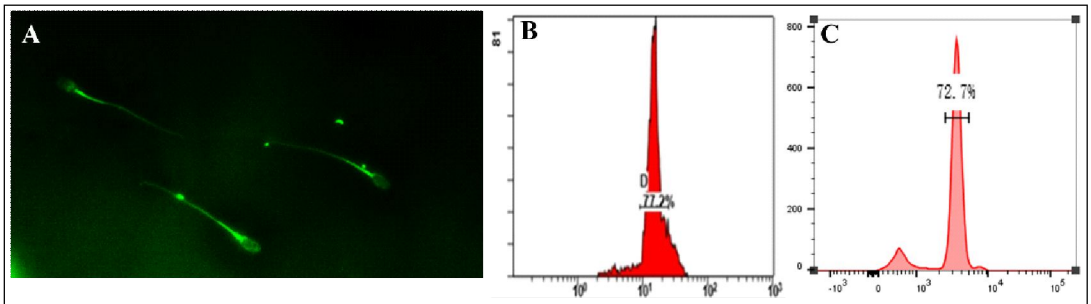
Sperm acrosome integrity

As shown in Fig 3A, the sperms having intact acrosome displayed without green or with only faint green fluorescence in the head (FITC-PNA$^-$) and sperms with damaged acrosome exhibited green head (FITC-PNA$^+$). The intact sperm acrosome proportion of the Congjiang Xiang pigs was 67.15±2.03%, which is lower than that of the Large White pigs (76.83±2.68%, P<0.05). The integrity of the sperm acrosome is the key to successful fertilization and proteolytic enzymes (acrosin) exist at the top of the sperm acrosome is closely related to the fertilization process (Sironen *et al*., 2010), which participates in the whole process of fertilization. Acrosome integrity was one of the sperm attributes which was used to distinguish between high and low-fertility bulls and sperm acrosome integrity could serve as a fertility biomarker (Bernecic *et al*., 2021; Gonzalez *et al*., 2020). The low intact sperm acrosome proportion may be one of the reasons that the reproductive ability of the Congjiang Xiang pigs was weaker than the Large White pigs.

There were no significant correlations between the FCM results and sperm forward motion and sperm vitality results of the two breeds. There was a positive correlation between the plasma membrane integrity and mitochondrial activity in the Congjiang Xiang pigs. An insignificant correlation between the plasma membrane integrity, mitochondrial activity and acrosome integrity was observed in the Large White pigs (date not shown).

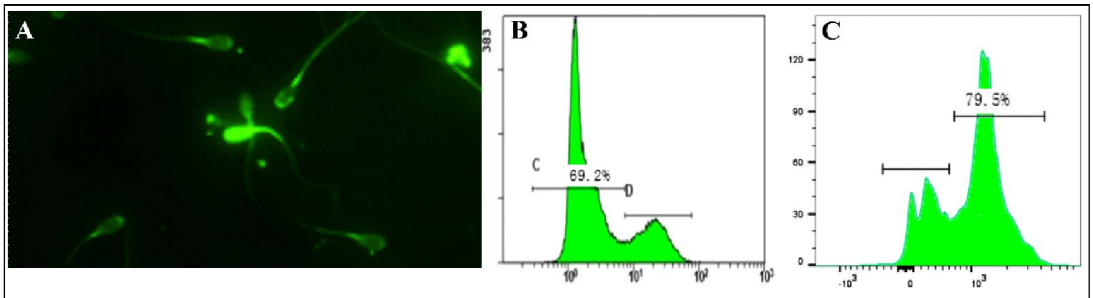
Sperm Rh123/PI double staining

Rh123/PIdouble staining can detect the integrity of plasma membrane and mitochondrial function of sperm at the same time. Under the fluorescence microscope, after Rh123/PIdouble staining, sperms exhibited different states (Fig 4). By FCM, it was found that there were significant differences in the percentage of Rh123$^+$/PI$^-$ and Rh123$^-$/PI$^-$ sperm between Congjiang Xiang pigs and the Large White pigs (P<0.05) (Fig 4A, B). The percentage of Rh123$^+$/PI$^-$ from Congjiang Xiang pigs was 70.27±1.76%, which is higher than that from the Large White pigs (61.32±0.86%, P<0.05). The percentage of Rh123$^-$/PI$^-$ from the Congjiang Xiang pigs was 17.81±0.53%, which is lower than that from the Large White pigs (24.45±1.42%, P<0.05). There were no significant differences between PI$^-$ single staining and Rh123/PI$^-$ double staining in detecting sperm membrane quality (P>0.05). There were no significant differences between single Rh123$^+$ and double Rh123$^+$/PI in detecting sperm mitochondrial activity (P>0.05). Unlike single staining results, the Rh123/PI double staining appears to provide new evidence that there is an advantage of Congjiang Xiang pigs in the rate of plasma membrane integrity with normal sperm mitochondrial



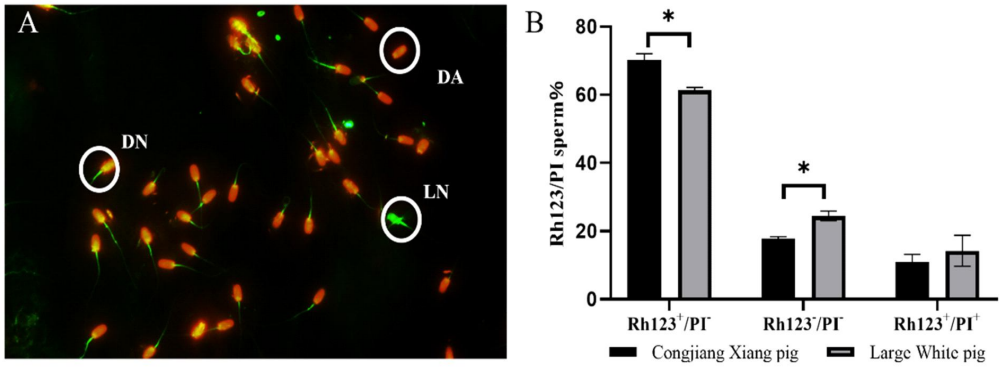
A: Sperms with normal mitochondrial function was stained by Rh123 (×400). B-C: Mitochondrial function in sperm of Congjiang Xiang pigs (B) and Large white pigs (C).

Fig 2: Sperm Rh123 fluorescent staining and FCM result.



A: After staining with FITC-PNA, the sperm without acrosome staining or with only faint green fluorescence was the sperm with intact acrosome. B-C: Intact acrosome in sperm of Congjiang Xiang pigs (B) and Large White pigs (C). FITC-PNA$^+$ showed sperms with the damaged plasma membrane and FITC-PNA$^-$ sperms had intact plasma membranes.

Fig 3: Sperm FITC-PNA fluorescent staining.



A: The Rh123/PI double staining of the sperm; B: Two breeds Rh123/PI double staining FCM result. DN: Dead sperm with normal mitochondria functioned; DA: Dead sperm with abnormal mitochondria functioned; LN: Live sperm with normal mitochondria functioned.

Fig 4: Sperm Rh123/PI fluorescent staining.

function compared with Large White pigs. The Rh123/PI double staining offers a more accurate and specific readout of the magnitude of the membrane integrity and mitochondrial activity in the boar sperm providing a method of reducing the sample needed for detecting boar sperm quality. The JC-1/PI double staining was used to detect sperm mitochondrial function in goats and bovine has been described before. However, this double staining has defects. In JC-1/PI double staining, the FCM cannot depart the red fluorescence of dead sperm and the red-orange of the sperm with high MMP affects the accuracy of the FCM result (Chai *et al*., 2011). Therefore, the Rh123/PI double staining was selected to improve the accuracy of sperm evaluation.According to previous studies, the Rh123/PI double staining can detect the bulls sperm mitochondrial function and plasma membrane integrity simultaneously(Johnson *et al*., 2020). It provides a simpler method for analyzing the relationship between sperm mitochondrial function, plasma membrane integrity and oligoasthenozoospermia (Zou *et al*., 2010).

CONCLUSION

Our study suggests that the semen of the Congjiang Xiang pigs is less rich in sperm concentration and sperm with acrosome integrity than that collected from the Large White pigs. The rate of live sperm with the normal mitochondrial function of Congjiang Xiang pigs was significantly higher and the rate of necrotic sperms was lower than those of the Large White pigs. Rh123/PI dual fluorescent staining and FCM can provide reliable information to assess the sperm quality of boar and reveal differences in MMP. This study first investigated the semen characteristics of the Congjiang Xiang pigs with FCM. These findings can guide nucleus herds in developing the standards of semen processing in Congjiang Xiang pigs and give new insights into their conservation and utilization.

Conflict of interest

All authors declare that they have no conflict of interest.

REFERENCES

Garner, D., Thomas, C.A., Joerg, H., DeJarnette, J. and Marshall, C. (1998). Fluorometric assessments of mitochondrial function and viability in cryopreserved bovine spermatozoa. Biology of Reproduction. 57(6): 1401-1406.

Iafrate, A., Novara, G., Galfano, M., Berto, V., Cavalleri, M., Artibani, S. and Ficarra, W. (2008). Improvement of seminal parameters and pregnancy rates after antegrade sclerotherapy of internal spermatic veins. European Urology Supplements. 7(3): 128.

Li, H., Zhang, X.G., Fang, Q., Liu, Q., Du, R.R., Yang, G.S., Wang, L.Q. and Hu, J.H. (2017). Supplemental effect of different levels of taurine in Modena on boar semen quality during liquid preservation at 17°C. Animal Science Journal. 88(11): 1692-1699.

Siqueira, A.P., Wallgren, M., Hossain, M.S., Johannisson, A., Sanz, L., Calvete, J. and Rodriguez, M.H. (2011). Quality of boar spermatozoa from the sperm-peak portion of the ejaculate after simplified freezing in MiniFlatpacks compared to the remaining spermatozoa of the sperm-rich fraction. Theriogenology. 75(7): 1175-1184.

Sderquist, L., Janson, L., Larsson, K. and Einarsson, S. (1991). Sperm Morphology and Fertility in A. I. Bulls. Journal of Veterinary Medicine Series A. 38(7): 534-543.

Aksoy, E., Aktan, M., Duman, S. and Cuce, G. (2012). Assessment of spermatozoa morphology under light microscopy with different histologic stains and comparison of morphometric measurements. International Journal of Morphology. 30(4): 1544-1550.

Andrade, A.D., Arruda, R.D., Celeghini, E., Nascimento, J., Martins, S., Raphael, C.F. and Moretti, A.S. (2010). Fluorescent stain method for the simultaneous determination of mitochondrial potential and integrity of plasma and acrosomal membranes in boar sperm. Reproduction in Domestic Animals. 42(2): 190-194.

Bernecic, N.C., Donnellan, E., O'Callaghan, E., Callaghan, E., Kupisiewicz, K., O'Meara, C., Meara, C., Weldon, K., Lonergan, P., Kenny, D.A. and Fair, S. (2021). Comprehensive functional analysis reveals that acrosome integrity and viability are key variables distinguishing artificial insemination bulls of varying fertility - Science Direct. Journal of Dairy Science. 104(10): 11226-11241.

Broekhuijse, M., Sostaric - Zandee, E., Feitsma, H. and Gadella, B. (2012). Relation of flow cytometry sperm integrity assessments with boar fertility performance under optimised field conditions. Journal of Animal Animal Science. 90(12): 4327-36. doi: 10.2527/jas.2012-5040.

Chai, J., Gao, R.M., Zhang, J.X., Meng, K., Wang, H.M., Zhao, Y.H., Wang, Z.X., Zhang, W.G. and Jin-Quan, L.I. (2011). Detection of goat epididymal sperm mitochondrial membrane potential by jc-1 single staining and jc-1/pi co-staining method of fluorescent probe. Hubei Agricultural Sciences. 50(12): 2503-07+18.

Franken, D.R. (2015). Semen analysis workshops: 17 years' experience. Facts Views and Vision in Obgyn. 7(3): 173-75.

Freitas, M., Vijayaraghavan, S. and Fardilha, M. (2017). Signaling mechanisms in mammalian sperm motility. Biology of Reproduction. 96(1): 2-12.

Gong, T., Wang, W.Y., Xu, H.Q., Yang, Y., Chen, X., Meng, L.J., Xu, Y.J., Li, Z.Q., Wan, S.F. and Mu, Q. (2021). Longitudinal expression of testicular TAS1R3 from prepuberty to sexual maturity in congjiang xiang pigs. Animals. 11: 437.

Gonzalez-Castro, R. and Herickhoff, L. (2020). Flow cytometric viability and acrosome integrity assessment in cooled boar sperm using SYBR14 or Hoechst-33342. Anim. Reprod. Sci. 220(106398).

Guo, H.Y., Wang, J.R., Sun, L.Z., Jin, X.H., Shi, X.Y., Lin, J.Y., Yue, S.L. and Zhou, J.B. (2021). Effects of astaxanthin on plasma membrane function and fertility of boar sperm during cryopreservation. Theriogenology. 164: 58-64.

Hadi, H. and Ali, R. (2021). WHO Laboratory Manual for the Examination and Processing of Human Semen Fifth Edition.

Holden, S., Malama, E., Siuda, M., Bollwein, H. and Butler, S. (2020). *In vitro* comparison between sex-sorted and conventional bovine sperm employing multicolour flow cytometry. Animal Reproduction Science. 220: 106427.

Jäkel, H., Henning, H., Luther, A.M., Rohn, K. and Waberski, D. (2021). Assessment of chilling injury in hypothermic stored boar spermatozoa by multicolor flow cytometry. Cytometry Part A. 99(10): 1033-41.

Johnson, C., Dance, A., Kovalchuk, I., Kastelic, J. and Thundathil, J. (2020). Enhanced pre-pubertal nutrition upregulates mitochondrial function in testes and sperm of post-p ubertal Holstein bulls. Scientific Reports. 10(1): 2235.

Johnson, L., Weitze, K.F., Fiser, P. and Maxwell, W.M.C. (2000). Storage of boar semen. Animal reproduction Reproduction Science. 62(1-3): 143-172.

Kommisrud, E., Paulenz, H., Sehested, E. and Grevle, I. (2002). Influence of boar and semen parameters on motility and acrosome integrity in liquid boar semen stored for five days. Acta veterinaria Scandinavica. 43(1): 49-55.

Li, Y., Li, J., Zhang, Q., Wang, Q., Guo, M., Li, Q. and Wei, G. (2021). The effects of negative pressure on the gene expression of motility related proteins in boar spermatozoa during liquid storage at 17°C. Indian Journal of Animal Research. 55(5): 511-516. doi: 10.18805/IJAR.B-1337.

Lopes, S., Rosa, H. and Chaveiro, A. (2021). Influence of different freezing curves on the acrosome integrity of male goat sperm cells. American Journal of Animal and Veterinary Sciences. 16(1): 56-61.

Lopez, R.A., Soom, A., Arsenakis, I. and Maes, D. (2017). Boar management and semen handling factors affect the quality of boar extended semen. Porcine Health Management. 3(1): 15. DOI: 10.1186/s40813-017-0062-5.

Mcpherson, F.J., Nielsen, S.G. and Chenoweth, P.J. (2014). Seminal factors influencing return to estrus in female pigs following artificial insemination. Animal Reproduction. 11(1): 24-31.

Meng, L.J., Wang, W.Y., Xu, Y., Gong, T. and Yang, Y. (2020). Postnatal differentiation and regional histological variations in the ductus epididymidis of the Congjiang Xiang pig. Tissue and Cell. 67: 101411.

Murphy, M. (2016). Understanding and preventing mitochondrial oxidative damage. Biochemical Society Transactions. 44(5): 1219-1226.

Nag, P., Kumaresan, A., Sivamanikandan, A., Manimaran, A. and Ramesha, K. (2021). Sperm phenotypic characteristics and oviduct binding ability are altered in breeding bulls with high sperm DNA Fragmentation index. Theriogenology. 172: 80-87.

Rebelo, S., Cristo, M., Ramalho, S.J. and Amaral, S. (2021). Mitochondria-targeted compounds to assess and improve human sperm function. Antioxidants and Redox Signaling. 37(7-9): 451-80.

Sandoval, V.L., Dumorne, K., Contreras, P., Farias, J.G., Figueroa, E., Risopatron, J. and Valdebenito, I. (2021). Cryopreservation of coho salmon sperm (*Oncorhynchus kisutch*): Effect on sperm function, oxidative stress and fertilizing capacity. Aquaculture. 533(3): 736151.

Singh, M., Patra, M.K., Rajkhowa, D.J., Ngullie, E. and Mollier, R. (2019). Artificial insemination in pig: Technology for producing quality piglets in Nagaland. 69(7): 31-32.

Sironen, A., Uimari, P., Nagy, S., Paku, S. and andersson, M. and Vilkki, J. (2010). Knobbed acrosome defect is associated with a region containing the genes STK17b and HECW2 on porcine chromosome 15. BMC Genomics. 11(1): 699.

Vašíèek, J., Balazi, A., Svoradová, A., Vozaf, J., Dujíèková, L., Makarevich, A., Bauer, M. and Chrenek, P. (2022). Comprehensive flow-cytometric quality assessment of ram sperm intended for gene banking using standard and novel fertility biomarkers. International Journal of Molecular Sciences. 23(11): 5920. doi: 10.3390/ijms23115920.

Wolf, J., Smital, J. (2009). Quantification of factors affecting semen traits in artificial insemination boars from animal model analyses. Journal of Animal Science. 87(5): 1620-1627.

Wysokiñska, A. and Szablicka, D. (2021). Integrity of sperm cell membrane in the semen of crossbred and purebred boars during storage at 17°C: Heterosis Effects. Animals. 11(12): 3373. https://doi.org/10.3390/ani11123373.

Zhu, Z.D., Li, R.N., Li, Y.H., Lv, Y.H., Zeng, W.X. (2019). Melatonin protects rabbit spermatozoa from cryo-damage *via* decreasing oxidative stress. Cryobiology. 88: 1-8.

Zou, T., Liu, X., Ding, S. and Xing, J. (2010). Evaluation of sperm mitochondrial function using rh123/PI dual fluorescent staining in asthenospermia and oligoasthenozoospermia. Journal of Biomedical Research. 24(5): 404-410.