ORIGINAL ARTICLE

Veterinary Research Forum. 2021; 12 (3) 267 – 272

doi: 10.30466/vrf.2020.108342.2570

Journal Homepage: vrf.iranjournals.ir

Veterinary Research Forum

Potential fertilizer of spermatozoa in porcine epididymal tail post-orchiectomy

Mariela Adriana Ydiaquez-Miranda[1], José Antonio Herrera-Barragán[2], Miguel González-Lozano[2,3], Alejandro Ávalos-Rodríguez[2]*

[1] Doctorate in Biological and Health Sciences, Metropolitan Autonomous University, Mexico City, Mexico; [2] Department of Agricultural and Animal Production, Metropolitan Autonomous University-Xochimilco, Mexico City, Mexico; [3] Center for Teaching, Research and Extension in Swine Production (CEIEPP), Faculty of Veterinary Medicine and Animal Production, National Autonomous University of Mexico, Jilotepec, Mexico.

Article Info

Article history:

Received: 22 May 2019
Accepted: 24 April 2020
Available online: 15 September 2021

Keywords:

Fertilization
Phospholipid asymmetry
Sperm
Swine

Abstract

The aim of this study was to determine the potential fertilizing of spermatozoa from the epididymal tail in different periods of time post-orchiectomy (P-OQ). Therefore, the study was approached in two stages. In the first stage, the orchiectomy was performed in 30 adult pigs. The testicles were stored at 5.00 ˚C in physiological saline solution for 5, 24, 48, 72, 96 and 120 hr. The spermatozoa were obtained by backflushing the vas deferens. The spermogram and fluorometric study were performed for each sample to evaluate the exposure of phosphatidyl-serine (PS) and acrosome reaction (AR). The second stage included the fertilization test, 16 prepubertal sows were selected, after synchronizing the oestrous cycle and the post-cervical artificial insemination was performed with the refrigerated sperm samples from each P-OQ time. The percentage of live sperm remained without significant changes until 96 hr P-OQ. An increase in the percentage of spermatozoa that showed a PS exposure was observed. The premature AR was evident after 72 hr. Considering that the artificial insemination was performed ensuring a minimum number of live sperms, no significant differences were observed in the number of embryos and corpora lutea. The results indicated that pig sperm collected from the epididymal tail P-OQ and stored for 5 and up to 72 hr at 5.00 ˚C had viable characteristics and maintained their fertilization ability. However, there was an increase in the loss of phospholipid asymmetry of the plasma membrane as time increased (72 and 96 hr), therefore, sperm viability was decreased.

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Introduction

In the epididymal tail, the sperm remain alive until ejaculation, there are optimal conditions to preserve their fertilizing ability. Therefore, the recovery and preservation of epididymal tail sperm constitutes an alternative, not only for the conservation of genetic material from species with high zootechnical value, but also for the recovery of threatened species. Therefore, the use of spermatozoa obtained from epididymal tail by backflushing method is an essential tool to preserve the genetic potential of high value domestic animals, as well as wildlife animals that are in danger of extinction.[1] When the animals were injured, died or unable to obtain sperm in a conventional way or required castration,[1] therefore, the recovery of spermatozoa after the death of the animal will enable us to exploit and preserve genetic material.[2] These spermatozoa may be cooled down for later use, however, sperm quality may decline during the cooling period. The cooling process promotes decreased sperm metabolism, which extends the viability, but also affects the quality of the semen.[3]

Epididymal tail sperm have also been used in humans when undergoing reproductive assistance.[4] Previous studies have shown the viability of epididymal tail sperm of various species. In most cases, sperms are obtained after sacrifice or orchiectomy, from epididymis that have been stored under cooling conditions (5.00 ˚C) at different periods of time.[5] For example: domestic cats,[3] rat,[6] goats,[7] red Iberian deer,[8] felines,[9] canines,[10] bulls,[2,11] white-tailed deer,[12] European bison,[13] and pigs.[14,15] Once epididymal tail spermatozoa are obtained, they can be used in biotechnologies such as artificial insemination (AI) and *in vitro* fertilization (IVF). Although the *in vitro* embryonic

***Correspondence:**

Alejandro Ávalos-Rodríguez. PhD
Department of Agricultural and Animal Production, Metropolitan Autonomous University-Xochimilco, Mexico City, Mexico
E-mail: avalosr@correo.xoc.uam.mx

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production with spermatozoa in epididymal tail stored at 5.00 ˚C at various periods of time, has been shown in different animal species.[5] No reports of the potential fertilization on swine sperm recovered from epididymal tail at different periods of time P-OQ were observed.

Given all that, the purposes of this study were to evaluate the effect of P-OQ undercooling conditions by 5, 24, 48, 72, 96 and 120 hr according to the quality of spermatozoa obtained from epididymal tail in swine and to measure its fertilizing potential.

Materials and Methods

Reagents. The chemicals were acquired from the following sources: D-glucose, $NaHCO_3$, EDTA, citrate sodium, bovine serum albumin (BSA), HEPES, $CaCl_2$, L-cysteine, paraformaldehyde, chlortetracycline (Sigma, St. Louis, USA). Lincomycin and spectinomycin from Zoetis (Madrid, Spain). Xylazine and Zoletil® from Virbac Laboratories (Carros, France). Equine chorionic gonadotropin plus human chorionic gonadotropin from Intervet (Mexico City, Mexico). Annexin V FITC Conjugate from (Sigma). All other analytical grade chemicals were obtained from Merck (Darmstadt, Germany).

Biological material. Thirty adult pigs weighted 150 kg proven fertility and 16 prepubertal sows between 80.00 and 100 kg (six months old) from Center for Teaching, Research and Extension in Swine Production located in Jilotepec, Mexico State, Mexico, were used. All the animals remained clinically healthy and under the conditions of care as recommended by NOM-033-ZOO-1995.[16] This study was carried out in two phases. In the first one, spermatozoa of the epididymis were obtained and analyzed. In the second stage, their fertilizing ability was evaluated.

Surgical intervention. For the performance of orchiectomies from anesthetic protocols in boars xylazine and Zoletil® 100 by deep intramuscular injection (4.00 mg kg^{-1} and 2.20 mg kg^{-1}, respectively) were used according to the species and met the requirements of the American Association of Veterinary Medicine (AVMA). Immediately after orchiectomy, each testicle with its respective epididymis and vas deferens were placed into plastic bags containing 20.00 mL of physiological saline solution and stored at 5.00 ˚C for 5, 24, 48, 72, 96 and 120 hr.

Sperm retrieval from epididymis by the back-flushing method. After removing the serous cover and connective tissue from the testis, epididymis and vas deferens were separated. The sperm cells were recovered according to the backflushing method described by Ponglowhapan and Chatdarong.[17] Briefly, the caudal portion of the epididymis was cannulated and 50.00 mL of cold medium (5.00 ˚C) was administered (0.144 M D-glucose, 14.20 mM $NaHCO_3$, 8.20 mM EDTA, 27.20 mM citrate sodium, 44.20 mM BSA, 37.70 mM HEPES, 20.60 mM Tris. Lincomycin and spectinomycin 1:2, 1.00 g L^{-1},[18] with an osmolarity of 309 mOsm and pH 6.80), from the lumen of the vas deferens through the epididymal tail.

Semen testing. To estimate the live spermatozoa, immediately after retrieval, a supravital staining of Eosin-Nigrosin (Hycel Reactivos Químicos, Mexico City, Mexico) was performed.[19] Sperm sample was mixed with eosin-nigrosine staining (1:1) and smear was prepared. The smear was analyzed with a light microscopy with a 40× objective, which was described by Ávalos *et al.*[19,20] Two hundred spermatozoa were counted in at least five fields, and the percentage of cells stained was obtained. Sperm without staining (white) were considered alive or with an intact plasma membrane and pink sperm were considered as dead and with membrane damaged.[3] The determination of the sperm concentration was performed using a Neubauer chamber (Neubauer improved bright-line; Superior-MarienFeld, Lauda-Königshofen, Germany), the seminal samples were previously diluted with distilled water using a pipette for BLAUBRAND® white blood cells according to the formula of Ávalos *et al.*[19] Progressive mobility was evaluated by microscopic visual estimation (40×) of 200 spermatozoa and the percentage of mobile cells was obtained in 10.00 µL sample.[19]

Phospholipid asymmetry. Phosphatidylserine (PS) exposure in the membrane was used to evaluate the phospholipid asymmetry. Twenty microliters of Annexin-V labeled with fluorescein isothiocyanate (10.00 µg mL^{-1}) in binding buffer (10.00 mM HEPES/NaOH, (pH 7.40), 140 mM NaCl, 4.00 mM $CaCl_2$) was added to an aliquot of 20.00 µL (150,000 cells). The samples were incubated for 15 min at room temperature under dark conditions, then they were analyzed under a Zeiss Axiovert 100 M fluorescence microscope (at 488 nm excitation and 530 nm emission; Zeiss, Munich, Germany) at 40× magnification.[19] After examining at least 200 sperm per field (bright field), green sperm (Annexin V-positive) were counted and the result was recorded as a percentage.

Evaluation of acrosomal reaction. According to Méndez *et al.,* 100 µL of the sperm sample was placed in a vial and 100 µL of CTC solution (20.00 mM Tris-HCl, 130 mM NaCl, 5.00 mM L-cysteine and 750 mM chlortetracycline) was added and mixed.[21] After 10 sec, the reaction was stopped with 12.50% paraformaldehyde (1:1) in 0.50 M Tris-HCl. At least 200 spermatozoa per field (five fields) were reviewed and the spermatozoa showing acrosomal reaction (without fluorescence in the acrosomal region) were counted and the result was recorded as a percentage.

Preparation of sperm samples. A dilution of the sample was performed to ensure a concentration of 1.50 × 10^9 viable sperm in 50.00 mL with the medium used for backflushing.[22] The samples were placed in seminal packaging tubes (Minitube, Verona, USA) with an ability of 95.00 mL. Once the dilutions were made, they were kept at 15.00 ˚C until their use.

Intrauterine artificial insemination. Sixteen sows, intended to trace, were selected for oestrous stimulation and detection using an exposure protocol in boards. To synchronize an oestrous, 400 IU of equine chorionic gonadotropin plus 200 IU of human chorionic gonadotropin were administered via intramuscular route to each sow. The AI was performed three days after the synchronization. The animals were divided into four groups of four sows each and were inseminated with the epididymal tail sperm samples, previously stored for 5, 24, 48 and 72 hr. The AI post-cervical was performed, twice per oestrous cycle (morning and afternoon), with a Minitube® catheter with cannula (Minitube).

Embryo collection. Five days after the AI, females of each group were sacrificed (as indicated in NOM-033-ZOO-1995),[16] to recover the embryos (morula or blastocysts).[22,23] The embryos were recovered by washing off uterine horns with 150 mL of phosphate-buffered saline, the blastocysts were removed by gravity making a slight massage from uterine horns to the body of the uterus.[24] The blastocysts were maintained for 5 min at 38.00 ˚C in a Petri dish and analyzed under a stereoscopic microscope.

Statistical analysis. The Proc GLM in SAS package (version 9.2; SAS Institute, Cary, USA) was used. The results were analyzed by ANOVA for a generalized linear mixed model under the values of the spermiogram (progressive motility and viability) and the PS exposure as dependent variables, while the hours of post-orchiectomy collection were independent variables. Number of embryos recovered and the time post-orchiectomy were considered as covariates. The Pearson correlation test was used to analyze the relationships between the different variables. The differences between the post-orchiectomy times were analyzed by the Tukey comparison test with $p < 0.05$.

Results

The animals were anesthetized (as previously described in Materials and Methods) to perform the orchiectomy and the recovery of the animals was carried out without setbacks.

Figure 1 shows Eosin-Nigrosin staining (A, vital staining) and PS exposure (B, Annexin V-Fluos positive) from epididymal tail sperm obtained post orchiectomy and stored at 4.00 ˚C for 96 hr. As the storage time of the P-OQ testicles was increased, the percentage of live spermatozoa and progressive mobility was decreased significantly ($p < 0.05$), from 72 onwards and continued decreasing to 96 and 120 hr. Regarding the PS exposure percentage, it was increased significantly ($p < 0.05$) from 48 hr. and continued during the following P-OQ times (72, 96 and 120 hr). The acrosome reaction was 2.20 and 2.90 higher at 72 and 120 hr when compared to 5 hr of storage (Table 1).

The conception rate was 100% with epididymal tail sperm stored in refrigeration for 5, 24, 48 and 72 hrs. Presence of blastocysts and corpus luteum were demonstrated in all study times (Figs. 1D and 1E). No significant statistical differences were observed between the number of *corpora lutea* or embryos at different periods of times ($p < 0.05$). However, the number of embryos was lower when using refrigerated spermatozoa in epididymal tail for up to 72 hr (Table 2).

Discussion

The results of this study showed that sperm obtained from epididymal tail and stored for 2 days at 4.00 ˚C could be used for artificial insemination in sows with a high percentage of fertilization, as evidenced by the presence of five day-old embryos.

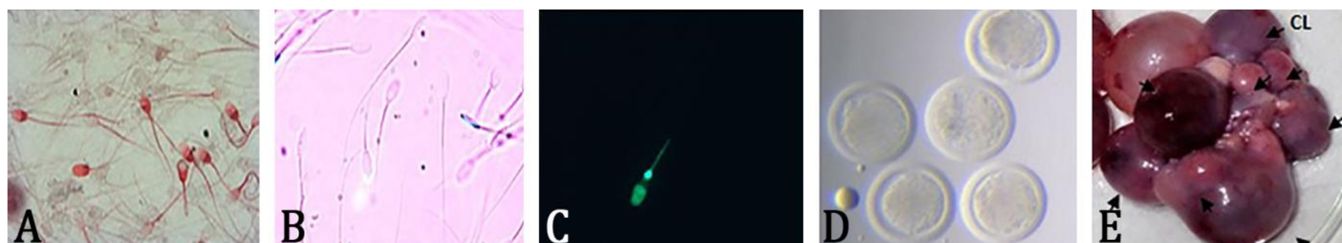

Fig. 1. Sperm P-OQ stored at 4.00 ˚C for 96 hr. **A)** Eosin-Nigrosin staining; **B)** Bright field; C**)** Phosphatidylserine exposure (40×); **D)** Blastocysts (200×), and **E)** Swine corpus luteum (CL: arrows, 40×) inseminated with epididymal tail sperm P-OQ stored at 4.00 ˚C for 72 hr.

Table 1. Effect of post-orchiectomy time on sperm parameters.

Time (hr)	Viability (%)	Progressive mobility (%)	Phosphatidylserine exposure (%)	Premature acrosomal reaction (%)
5	91.20 ± 4.61^{a}	85.20 ± 5.98^{a}	2.70 ± 1.25^{d}	20.00 ± 1.05^{a}
24	84.40 ± 14.86^{ab}	82.90 ± 7.11^{ab}	3.90 ± 1.20^{cd}	2.20 ± 1.03^{ab}
48	83.40 ± 5.38^{ab}	79.70 ± 4.30^{abc}	4.80 ± 1.32^{c}	3.10 ± 0.99^{ab}
72	78.80 ± 4.69^{b}	75.50 ± 4.30^{cd}	6.80 ± 1.03^{b}	4.20 ± 1.40^{b}
96	74.30 ± 6.00^{bc}	70.20 ± 3.26^{d}	7.70 ± 1.95^{ab}	3.20 ± 1.03^{ab}
120	65.30 ± 9.17^{c}	60.10 ± 6.90^{e}	10.00 ± 2.87^{a}	4.90 ± 1.45^{c}

[abcd] Different letters indicate significant statistical difference between the data of the same column ($p < 0.05$).

Table 2. Corpus Luteum and embryos recovered from sows inseminated with epididymal tail sperm.

Time (hr)	No. corpus luteum	No. embryos
5	21.25 ±2.75	17.00 ± 2.16
24	19.50 ± 1.73	18.75 ± 2.22
48	21.00 ± 2.58	17.50 ± 2.65
72	20.25 ± 2.22	14.50 ± 2.08

No significant statistical differences were found between the data of the same column ($p > 0.05$).

Spermatozoa in epididymal tail possess higher values of progressive motility, viability and ability to penetrate an oocyte. An optimal number of piglets can be obtained from frozen epididymal sperm.[25,26,27] Therefore, it has been suggested that cryopreserved epididymal spermatozoa could be a practical option to produce swine.[27]

In this study, the fertilizing ability of porcine spermatozoa obtained from epididymal tail and stored at 5.00 ˚C at different POQ times (5, 24, 48.72, 96 and 120 hr.) was evaluated. Over time, researchers have tried to identify which sperm possess the most appropriate morphophysiological characteristics to fertilize the oocyte.[28,29] One of the characteristics that qualify the sperm as fertile is the membrane integrity. Therefore, one of the requirements for successful conception is that the sperm preserves the integrity and functionality of its membrane characterized by being asymmetric. Loss of phospholipid asymmetry can be evaluated by the exposure of phosphatidylserine (PS) on the outer side of the membrane, as it is normally confined to the inner leaflet. PS exposure can be detected with Annexin V-labeling. Although, it has been shown that during the capacitation process and acrosomal reaction, the loss of phospholipid asymmetry is a necessary event to perform fertilization,[28] when PS exposure occurs in sperm prior to AI, this could be the cause of capacitation (C) and premature acrosome reaction (AR), before its arrival with the oocyte, which could be a factor that would promote the fertilizing inability.

The results of this study showed an increase in the percentage of spermatozoa with PS exposed from epididymal caches stored at 5.00 ˚C for 96 and 120 hr POQ. Kotwicka *et al.* proposed that, if the PS was on the outer side of the plasma membrane, before the inter-action of the sperm with the oocyte, the spermatozoa were susceptible to experiencing premature acrosome reaction, which would lose functionality.[29] During the sperm capacitation, PS exposure suggests a process of apoptosis. However, Martin *et al*.,[30] showed that loss of mitochondrial membrane potential, caspase activation, increased permeability of the plasma membrane and increased fragmentation of DNA (all signs of apoptosis), they are not observed in spermatozoa, even after 4 hr of incubation with calcium ionophore A23187. Therefore, the PS exposure in human sperm is mainly related to the acrosome reaction and not necessarily with apoptosis.[30,31] However, there are reports showing that with removing of spermatozoa with PS exposure, fertility potential improves in humans, and suggests that the PS exposure could become a prognostic marker of sperm fertility potential.[32]

The spermatozoa obtained from epididymis preserved at (5.00 ˚C), up to 72 hr. did not show changes that compromised their viability or progressive mobility. Thus, the integrity of the acrosomal membrane was maintained. These parameters increase the probability of success in fertilization. The storage temperature is an important requirement since the metabolism of sperm cells is reduced in terms of mitochondrial oxidative phosphorylation and glycolytic activities, causing a thermotropic trans-formation in the membrane phospholipids of the sperm cell resulting in a malfunction which cannot go back.[33] In addition, the plasma membrane has a relatively high content of polyunsaturated fatty acids and a low concentration of cholesterol, leading to an imbalance of sterol-phospholipid and loss of membrane stability due to a decrease in temperature, compared to what happens with spermatozoa of other mammals.[34] Turri *et al.*[7] state that sperm motility and progressive viability is assured when the testes are transferred and stored at 5.00 ˚C, up to a maximum of 48 hr. after death and at 4.00 ˚C up to 72 hr when cryopreservation is not possible.[35]

Studies conducted by Oh *et al.*[36] in porcine sperm indicated that the status of sperm ability was a useful predictor of its fertility and that it correlated positively with a litter size. These breakthroughs adhered to the findings of this study, where it was observed that as the POQ time was increased, the number of spermatozoa with PS exposure, premature AR and decrease of motility also were increased.[37] This would undoubtedly reduce the fertilizing ability. However, in our study, those spermatozoa preserved in the epididymal tail maintained their fertilizing ability for up to 72 hr.

The changes at the membrane level observed after 48 hr POQ can decrease the fertility rate. In this study, the success in the fertility rate among sows was probably due to the fact that an optimum concentration of viable sperm (30.00×10^6 / mL) was used 50.00 mL for artificial insemination according to Knox,[22] which guarantees high fertility rates.[38] Additionally, care was taken that the AI was at the correct time (21 days after the last oestrous cycle) and no reflux of semen was observed during insemination. Using this concentration of viable sperm and performing the post-cervical AI twice, it is possible to inseminate a certain number of females depending on the sperm quality, ensuring good fertility rates. However, when the storage time increases, the sperm quality decreases reflecting a decrease in the amount of dose. In this sense, it is important to mention that the shorter the storage time, the higher the sperm quality and the higher the number of insemination doses and the longer the storage time, the lower the sperm quality and the lower

the quantity of insemination. It is known that with a sample of fresh ejaculate, approximate 10-15 sows can be inseminated. [39] However, to keep the epididymis in cooling conditions for up to three days and recover sperm that is in the epididymal tail, could ensure the offspring of animals with high genetic value, when there is an unexpected death and there are no conditions to obtain a sample of ejaculate and cryopreserve them. Previous studies have shown that the *in vitro* embryonic production with epididymal spermatozoa is possible if the testicles are stored under cooling temperatures and reach the laboratory in less than 72 hr.[40,41]

We concluded that the quality of porcine spermatozoa obtained from the epididymal tail was decreased progressively, as the storage time was increased under cooling conditions (5.00 ˚C), likewise the exposure of PS was increased, which could play an important role as an early marker of sperm quality. The fertilizing ability of the spermatozoa is maintained even up to 72 hr after being stored under cooling conditions, so they can be used for AI post-cervical with a high percentage of fertility, as long as the minimum concentration of viable sperm is ensured.

Acknowledgments

We thank Center for Teaching, Research and Extension in Swine Production (CEIEPP), for providing support along the development of this study. We also thank CONACYT for the scholarship No. 425681 awarded to Mrs. Mariela Adriana Ydiaquez Miranda.

Conflict of interests

The authors declare that they have no competing interests.

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