Animal Science Papers and Reports vol. 39 (2021) no. 2, 119-129
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Preimplantation development of *in vitro* matured porcine oocytes fertilized with ejaculated and epididymal spermatozoa

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(Accepted May 4, 2021)

The aim of this study was to determine the quality of ejaculated and epididymal boar spermatozoa before and after capacitation and the development of *in vitro* matured porcine oocytes fertilized with these two types of spermatozoa. Fresh, ejaculated spermatozoa and spermatozoa from epidydimal tails were used. The following parameters of ejaculated spermatozoa (before and after capacitation) and epidydimal spermatozoa (after capacitation) were evaluated using SCA: motility, progressive motility, curvilinear velocity (VCL), straight-line velocity (VSL), hyperactivity and apoptotic-like changes. The motility and progressive motility of epidydimal spermatozoa (before capacitation) were assessed under a microscope. After *in vitro* fertilization and embryo culture the quality of obtained blastocysts was assessed. Before and after capacitation, statistically significant differences ($P<0.05$) were found when comparing the progressive motility of ejaculated (60.77 and 68.8%, respectively) and epididymal spermatozoa (30.0% and 55.7%, respectively). Moreover, after capacitation significant differences ($P<0.01$) between these two types of semen were recorded in VCL (49.2 and 83.3%, respectively) and hyperactivity (50.5 and 13.2%, respectively). However, there were no differences in the proportions of live and necrotic spermatozoa between ejaculated and epididymal spermatozoa before and after capacitation. The developmental potential of porcine embryos obtained after fertilization with ejaculated (53.7% of cleaved embryos and 25% of blastocysts) and epididymal spermatozoa (55.1 and 30.5%, respectively) was similar. A higher total number of cells per blastocyst was obtained after *in vitro* fertilization with ejaculated spermatozoa (mean 37.13±10.39, n=16) than with epididymal spermatozoa (mean 28.46±6.72, n=13) ($P<0.05$). In conclusion, boar spermatozoa - irrespective of their type (ejaculated or epididymal) - displayed a similar susceptibility to *in vitro* capacitation and *in vitro* fertilization.

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KEYWORDS: apoptosis / *in vitro* fertilization / *in vitro* culture / pig / spermatozoa

The effectiveness of *in vitro* fertilization (IVF) in pigs depends on many factors, including the type of spermatozoa, the quality of spermatozoa and the individual susceptibility of boar spermatozoa to biotechnological processes, such as capacitation *in vitro*. For the IVF procedure, ejaculated or epididymal fresh or frozen spermatozoa are used [Linh *et al.* 2018, Cho *et al.* 2020]. The success of the fertilization process depends to a high degree on the ability of spermatozoa to undergo capacitation and penetrate the oocyte [Soriano-Úbeda *et al*. 2019].

The basic evaluation of spermatozoa includes analysis of motility as well as the concentration and morphology of spermatozoa. Motility and hyperactivation are both important aspects of assessment used to describe the ability of spermatozoa to move in the female's reproductive system and to penetrate the oocyte. Progressive movement is an indicator of correct metabolism and intact membranes of spermatozoa. Taking into consideration the fact that a higher percentage of boar spermatozoa exhibit circular movement cmpared to those of other mammals, it is recommended to assess different forms of motility, including progressive motility [Johnson *et al.* 2000]. It is believed that assessment of motile spermatozoa is an indicator of their capability to fertilize and is useful when evaluating the reproductive effectiveness of boar spermatozoa that exhibit motility below 60% [Flowers 1997]. It has been demonstrated that curvilinear velocity (VCL) is highly correlated with *in vitro* fertilization effectiveness [Holt *et al.* 1994]. At the same time, the features of the amplitude of lateral head displacement (ALH) and beat cross frequency (BCF) are able to determine the ability of spermatozoa to penetrate the zona pellucida [Niżański *et al*. 2006]. The dependencies related to spermatozoon quality and its fertilization abilities require further study. The condition of the acrosomal membrane is an important spermatozoon quality parameter. Any integrity disorders or damage to the acrosomal membrane can result in a decrease of fertilization effectiveness. A distinctive characteristic of the early stages of apoptosis is connected with a small change in the permeability of the cell membrane. Damage to the external membrane can be identified with the YO-PRO-1 fluorochrome [Bryła and Trzcińska 2012]. Assessment of spermatozoa may also include the application of flow cytometry and measurement of bioluminescence [Gogol *et. al*. 2009, Szczęśniak-Fabiańczyk *et al*. 2019]. It is important to note that currently the problem of reproduction is dominated by issues related to the integrity of the genetic material of spermatozoa at the chromatin and DNA levels. Moreover, damage in the nucleoprotein structure is treated as an indicator of the biological value of spermatozoa. Practical methods for evaluating semen quality to predict male fertility are still being researched.

Despite many improvements in the procedure of *in vitro* fertilization of porcine oocytes, results can be achieved ranging from approximately 30 to 90% of potential zygotes [Jeon *et al.* 2014, Lee *et al*. 2018]. By modifying the *in vitro* porcine embryo culture medium, an increase in the number of developed embryos can be observed, but the proportion of produced blastocysts does not exceed 50% [Nguyen *et al*. 2017, Poniedziałek-Kempny *et al.* 2020].

Until now the susceptibility to capacitation and the *in vitro* fertilization abilities of ejaculated and epididymal spermatozoa has not been compared.

The aim of this study was to assess the quality (before and after capacitation) and fertilization ability of ejaculated and epididymal spermatozoa and to evaluate the development and quality of embryos obtained after *in vitro* fertilization.

Material and methods

All chemicals were obtained from Sigma Aldrich (Sigma Chemical, Saint Louis, Missouri, USA) unless stated otherwise. All experimental procedures used in this study were carried out in accordance with the European Directive 2010/63/EU and were approved by the 2nd Local Ethical Commission in Krakow (decision no. 1181/2015, May 21, 2015).

Ejaculated spermatozoa

Semen was collected from 28 boars of the Polish Landrace breed (n=6) or mixed breeds (crossbreeds Duroc x Pietrain; n=13) and hybrid (Maxter, Hypor; n=9) at various ages (from 9 months to 3.5 years) housed at the Boar Semen Collection Centre in Klecza Dolna. Semen, after collection and dilution with a commercial extender (Biosolwens Plus, Biochefa, Poland), was transported to the laboratory and evaluated under a biological microscope (Nikon, 029725, Japan) as well as using the Sperm Class Analyzer CASA system(SCA®, Version 5.0 Microptic, Barcelona, Spain). The following parameters of spermatozoa were evaluated with the SCA CASA system: motility, progressive motility, straight linear velocity (VSL), curvilinear velocity (VCL), beat cross frequency (BCF) and hyperactivity. The concentration of spermatozoa was evaluated under a microscope with a *Bürker* chamber.

Epididymal spermatozoa

Spermatozoa were collected from 34 epididymides of 17 boars of different breeds at 8-9 months of age after slaughter. They were aspirated from epididymal tails with a 20 µL automatic pipette, diluted with the Biosolwens Plus extender (Biochefa, Poland) and evaluated under a microscope. The following parameters were evaluated: motility, progressive motility and concentration. Because of technical difficulties related to the malfunction of the SCA system epididymal spermatozoa before capacitation were evaluated only under a microscope, whereas epididymal spermatozoa after capacitation were assessed by both methods.

Evaluation of apoptotic changes in ejaculated and epididymal spermatozoa using fluorochrome YO-PRO-1/PI staining

Spermatozoa were analyzed for apoptosis with the YO-PRO-1 fluorochrome (Vybrant Apoptosis Assay Kit #4, Molecular Probes, USA) [Trzcińska *et al.,* 2011]. Semen samples containing $1x10^6$ spermatozoa were placed in PBS and subsequently

centrifuged for 10 min at a speed of 300 g. After centrifugation, the supernatant spermatozoa were rinsed in a solution of PBS, 2 µL of YO-PRO-1 and 1 µL of propidium iodide (PI) and incubated at room temperature in darkness for 30 min. After incubation, 200 spermatozoa from each sample were assessed under a fluorescent microscope (Eclipse E600) with the following filters: 520 x 20 nm wavelength, green fluorescence detection of apoptotic cells stained with YO-PRO-1 and >620 nm wavelength, red fluorescence detection of necrotic cells stained with PI.

***In vitro* capacitation**

In vitro capacitation of ejaculated and epididymal spermatozoa was carried out in a modified mM199 medium [Yang *et al.,* 1999]. This medium was enriched with calcium chloride (2.92 mM), sodium pyruvate (0.91 mM), glucose (3.05 mM), Hepes (25 mM) and 10% BSA (Bovine Albumin Serum). The capacitation process was as follows: 1 mL of fresh spermatozoa was centrifuged for 25 s (6000 rpm). After isolating the supernatant, the remaining fraction was centrifuged twice in PBS with the addition of 0.001 g/mL BSA. The obtained spermatozoon fraction was diluted in a capacitation medium to obtain a concentration of 1 x 10^6 /mL. The spermatozoa were then incubated for 1 h at a temperature of 39°C in an atmosphere of 5% CO_2/O_2 and were evaluated under a microscope (Nikon, 029725, Japan) and the SCA analysis was carried out. The assessed parameters included motility, progressive motility, VSL, VCL, BCF, hyperactivity, concentration and apoptotic modifications.

Oocyte maturation *in vitro*

Immature porcine oocytes were obtained after slaughter from sow and gilt ovaries. Ovaries were transported to the laboratory in PBS at 30-35°C within 1 h of collection. After the ovaries had been washed 3 times in PBS supplemented with kanamycin, oocytes were obtained by aspiration or by cutting the ovaries. The TCM 199 (Tissue Culture Medium-199) manipulation medium supplemented with 10% FCS (Fetal Calf Serum) and 0.068 mg/mL kanamycin was used for aspiration of the follicular fluid containing the oocytes. The IVM procedure was described in detail in a previous study [Poniedziałek-Kempny *et al.* 2020]. After 42-44 h of culture, oocytes were evaluated morphologically under a stereomicroscope without contrast (Nikon, SMZ800N, Japan). Oocyte maturation was verfiried based on the expansion of cumulus oophorus cells as well as the presence of the first polar body in the perivitelline space that was identified after the removal of cumulus cells. Mature oocytes (metaphase II) were designated for *in vitro* fertilization.

***In vitro* fertilization**

The IVF procedure was described by Poniedziałek-Kempny *et al.* [2020]. Mature oocytes were placed in a 50 µL droplet of the mM199 medium supplemented with 10 mM caffeine. Fifty µL of the mM199 capacitation medium with a concentration of spermatozoa of 1x 10^6/mL were added to the droplet. Coincubation of gametes was

carried out at a temperature of 39°C in a 5% CO_2 atmosphere at maximum humidity for 4 h.

Embryo culture to the expanded blastocyst stage

The culture procedure was demonstrated in a previous study [Poniedziałek-Kempny *et al.* 2020]. Culturing took place at a temperature of 39°C in the NCSU-23 medium (North Carolina State University) in a 5% CO_2 atmosphere at maximum humidity. The culture duration was 6 to 8 days. Embryos were examined under a stereomicroscope (Nikon, SMZ800N, Japan), and only those meeting the standards of Grade 1 derived by the International Embryo Transfer Society [Van Soom and Boerjan, 2012] were used.

Evaluation of apoptosis and the number of cells in obtained blastocysts

Apoptosis in blastocysts was evaluated based on DNA fragmentation analysis with the TUNEL method [Brison and Schultz, 1997]. In the blastocyst the number of cells and the level of nuclear DNA fragmentation were assessed. The TUNEL method used in this study was described previously [Bryła *et al.* 2009]. The apoptotic index was calculated based on the ratio of the number of apoptotic nuclei per blastocyst to the total cell number per blastocyst.

Statistical analysis

The data on apoptotic changes in boar spermatozoa as well as parameters of motility before and after capacitation were analyzed by Student's t-test. Preimplantation development of embryos obtained after *in vitro* fertilization of porcine oocytes and the quality of blastocysts obtained after IVF with boar spermatozoa were analyzed by the Chi^2 test. Differences of $P<0.05$ were considered to be statistically significant, whereas differences of $P<0.01$ were considered to be highly significant.

Results and discussion

In our experiment it was observed that the proportion of boar spermatozoa displaying progressive motility were higher ($p<0.01$) in ejaculated than in epididymal spermatozoa (Tab. 1). Similar results for the motility of ejaculated spermatozoa were reported by Ramió-Lluch *et al*. [2012] and Matás *et al.* [2010]. Lower levels of motility and progressive motility of ejaculated spermatozoa were recorded by Gonzàlez-Abreu *et al*. [2017]. The results of our experiments regarding the motility of epididymal spermatozoa before capacitation were similar to those of Matás *et al*. [2010]. It is important to note that the epididymal spermatozoa (before capacitation) in our experiment were evaluated not by SCA, but under a microscope. An additional issue associated with analyzing and evaluating this type of spermatozoa is connected with the fact that it is not easy to obtain samples for experiments.

During the analysis of sperm motility before and after capacitation statistically significant differences ($P<0.05$) were observed after capacitation between ejaculated and epididymal spermatozoa (Tab. 1). Increases in the VSL, VCL and hyperactivation parameters in both types of spermatozoa after capacitation were noted. The observed changes in the motility parameters of capacitated spermatozoa may indicate a positive correlation of these parameters with hyperactivation. Similar results were recorded by Ramió-Lluch *et al.* [2012], who observed an increase in the abovementioned motility parameters after incubating ejaculated spermatozoa in a capacitation medium for 1 h. Mortimer [1997] also concluded that after hyperactivation of boar spermatozoa their VCL increased and VSL decreased. In our study the hyperactivity of ejaculated spermatozoa significantly increased ($P<0.01$) after *in vitro* capacitation compared to that before capacitation (Tab. 1). Our results confirm the hypothesis of Ramió-Lluch *et al.* [2012] that hyperactivated motility is one of the most characteristic properties connected to capacitation.

When comparing the capacitation abilities of ejaculated and epididymal spermatozoa, it has been demonstrated that even a short contact of spermatozoa with seminal plasma inhibits this process [Nagai 1996]. However, Rath and Niemann [1997] presented conclusions stating that because of their lack of plasma, epididymal spermatozoa had a better ability to fertilize than ejaculated spermatozoa. In our experiments we observed higher percentages of cleaved embryos and expanded blastocysts obtained as a result of *in vitro* fertilization with epididymal spermatozoa than with ejaculated spermatozoa (non-significant differences). Among the available publications there are no studies comparing the development of embryos obtained as a result of *in vitro* fertilization with fresh ejaculated and epididymal spermatozoa.

One of the criteria in the evaluation of spermatozoa is the level of apoptotic changes in spermatozoa [Chen *et al.* 2006]. In the early stages of

Table 1. Averages and standard deviations (in parenthesis) of selected motility parameters before and after capacitation of ejaculated and epididymal semen designated for IVF

Sperm type	Spermatozoa													
	before capacitation							after capacitation						
	motility (%)	concentr. ($1x10^9$/mL)	progressive motility (%)	VSL (μm/s)	VCL (μm/s)	BCF (Hz)	hyperactivity (%)	motility (%)	concentr. ($1x10^9$/mL)	progressive motility (%)	VSL (μm/s)	VCL (μm/s)	BCF (Hz)	hyperactivity (%)
Ejaculated (n=7)	70.0 (10.35)	26.0 (7.25)	60.77[a] (12.95)	48.12 (11.52)	45.79 (22.82)	7.04 (0.45)	14.81[i] (7.53)	68.8[c] (12.92)	3.9 (1.86)	61.7 (9.8)	57.7 (13.97)	49.2[e] (17.89)	6.8 (0.7)	50.5[g] (16.85)
Epididymal (n=4)	65.0 (3.54)	73.0 (6.08)	30.0[b] (3.54)					55.7[d] (16.43)	12.1 (8.78)	47.2 (20.13)	44.3 (11.70)	83.3[f] (9.76)	7.0 (0.54)	13.2[h] (8.75)

a,b – $P=0.0260$; c,d - $P=0.2165$; e,f – $P=0.0111$; g,h – $P=0.0046$; i,g – $P=0.0004$.

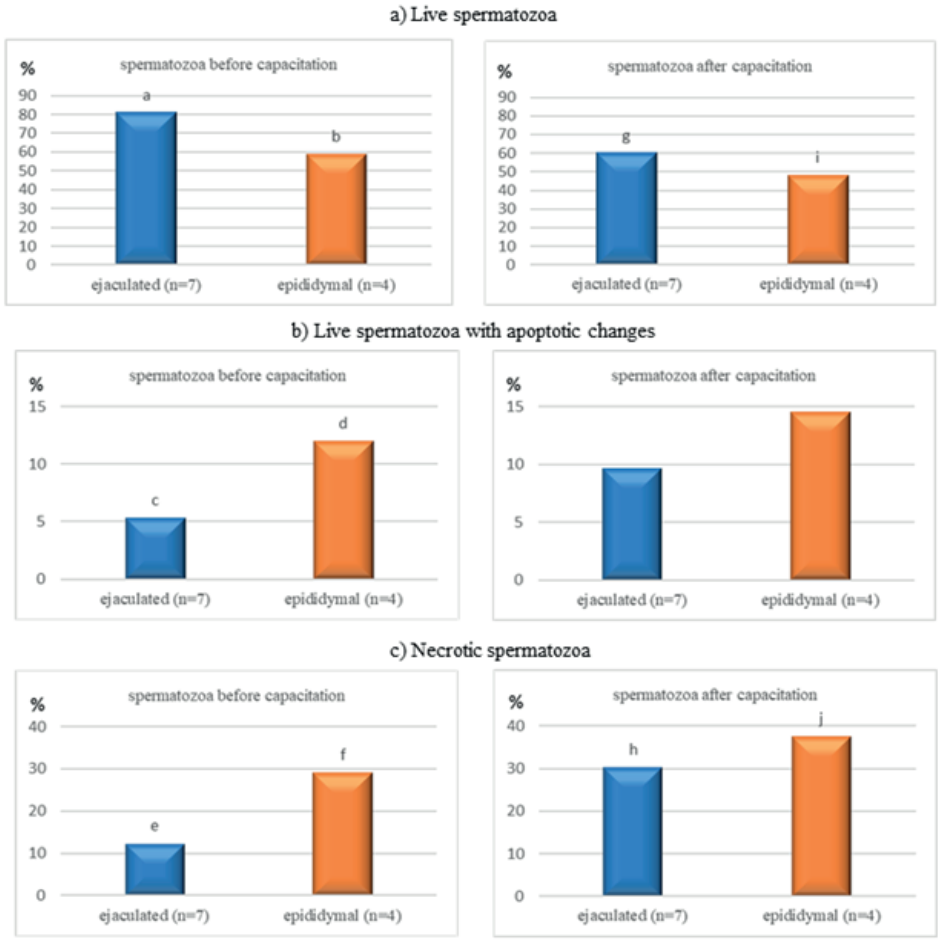

Fig. 1. Apoptotic changes in boar spermatozoa obtained from ejaculated and epididymal spermatozoa, designated for IVF.
a,b – P=0.0009; e,f – P=0.0003; a,g – P=0.0043; e,h - P=0.0030; c,d – P=0.0443; b,i – P=0.0417; f,j – P=0.0196.

apoptosis, a change occurs in the permeability of the cell membrane and the generated micropores can be identified with the use of fluorochrome YO-PRO-1 [Trzcińska *et al.* 2008, Bryła and Trzcińska 2012]. Therefore, in this study the evaluation of spermatozoa was expanded to include apoptotic changes in spermatozoa. Our results showed that compared to epididymal spermatozoa the level of apoptosis was lower ($p<0.05$) in ejaculated spermatozoa before capacitation. Contrary to these findings, Matás *et al*. [2010] recorded- lower levels of apoptotic changes before sperm capacitation in epididymal spermatozoa than in ejaculated spermatozoa. The reason for

our inferior evaluation results and higher apoptosis levels in epididymal spermatozoa may result from the young age of boars (8-9 months) and the fact that they were not used for tests in the past. However, there were no differences in the proportions of live and necrotic spermatozoa between ejaculated and epididymal spermatozoa before and after capacitation (Fig. 1).

In our study fresh ejaculated and epididymal boar spermatozoa were used for *in vitro* fertilization. However, the results of fertilization with frozen epididymal spermatozoa described by Jeon *et al*. [2014] were similar to our results with fresh epididymal spermatozoa with regard to the proportion of obtained blastocysts. The developmental potential of porcine embryos obtained after fertilization with ejaculated (53.7% of cleaved embryos and 25% of blastocysts) and epididymal (55.1 and 30.5% respectively) spermatozoa was comparable. Both fresh ejaculated spermatozoa

Table. 2. Preimplantation development of embryos obtained after fertilization of porcine oocytes withejaculated and epididymal spermatozoa

Spermatozoa type	Matured oocytes n (rep.)	Cleaved embryos *n* (%)	Morulae *n* (%)*	Expanded blastocysts *n* (%)*
Ejaculated	120 (5)	64 (53.7)	38 (59.4)	16 (25.0)
Epididymal	104 (4)	59 (55.1)	33 (55.9)	18 (30.5)

*In comparison to cleaved embryos.

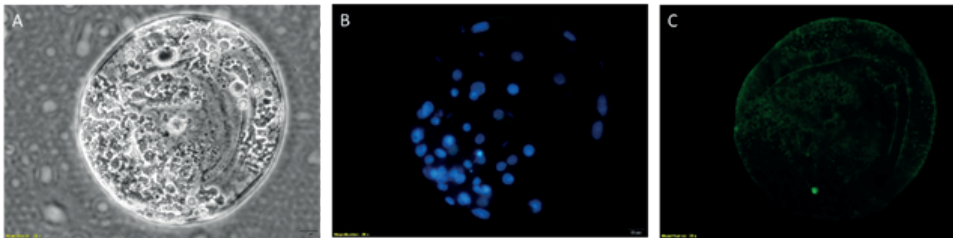

Fig. 2. An example porcine blastocyst obtained as a result of in vitro fertilization of mature oocytes with ejaculated semen. A: Light microscope image (20x). B: Fluorescent microscope image after DAPI staining (20x). Blue fluorescence indicates cell nuclei. C: Fluorescence microscope image after fluorescein staining (20x). TUNEL analysis reveals the green fluorescence of cell nuclei with DNA fragmentation.

and fresh spermatozoa obtained from epididymides demonstrated a similar *in vitro* fertilization ability (Tab. 2).

There are multiple ways of assessing the quality of embryos obtained in vivo and in vitro. The most important ones are based on the total number of cells in a blastocyst and the number of apoptotic nuclei (Fig. 2).

We detected a higher total number of cells in blastocysts obtained after in vitro fertilization with ejaculated spermatozoa than after in vitro fertilization with epididymal spermatozoa ($P<0.05$). Moreover, blastocysts obtained as a result of fertilization with epididymal spermatozoa demonstrated a lower level of apoptotic nuclei ($P<0.05$) compared to fertilization with ejaculated spermatozoa (Tab. 3).

Table 3. Quality of blastocysts obtained after IVF with ejaculated and epididymal spermatozoa

Spermatozoa type	Evaluated blastocysts *n*	Average cells number/ blastocyst (SD)	Average number of apoptotic nuclei / blastocysts (SD)	Apoptotic index (% TUNEL)
Ejaculated	16	37.13 (10.39)	1.75 (1.29)	0.05(0.03)
Epididymal	13	28.46(6.72)	0.69 (1.18)	0.03(0.05)
P-value		0.152	0.309	

Available publications do not present comparisons of blastocyst quality after fertilization with fresh ejaculated and epididymal spermatozoa. However, in comparative studies of fresh or frozen epididymal spermatozoa [Appeltant *et al.* 2015] better blastocyst quality was recorded after fertilization with fresh spermatozoa.

Our experiments demonstrated that among the two experimental sperm types, better motility parameters before capacitation were observed for ejaculated spermatozoa. Before and after capacitation statistically significant differences were found when comparing the progressive motility of ejaculated and epididymal sperm. The in vitro process of boar sperm capacitation leads to an increase in the proportion of necrotic spermatozoa and a decrease in the number of live spermatozoa (both ejaculated and epididymal spermatozoa) after capacitation compared to those before capacitation. We observed a similar in vitro fertilization ability of both ejaculated and epididymis boar spermatozoa. The developmental potential of porcine embryos obtained as a result of in vitro fertilization with ejaculated and epididymal spermatozoa was also similar. Porcine blastocysts obtained after in vitro fertilization with ejaculated spermatozoa showed a higher total number of cells in blastocysts than after in vitro fertilization with epididymal spermatozoa.

In conclusion, boar spermatozoa – irrespective of type (ejaculated or epididymal) – displayed a similar susceptibility to in vitro capacitation and in vitro fertilization.

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