For Theriogenology

Title: Application of a Microfluidic Sperm Sorter to the In-Vitro Fertilization of Porcine Oocytes Reduced the Incidence of Polyspermic Penetration

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Two figures and 4 tables

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Abstract

Microfluidic sperm sorter (MFSS) has been developed to isolate motile human spermatozoa by two laminar flows in the micro-channel without centrifugation. The present study was undertaken to apply MFSS to the porcine IVF. Boar sperm-rich fraction was diluted at 1 x 10^8 spermatozoa/ml with a diluter containing 20% seminal fluid. In the first experiment, when this diluted semen was flowed with modified TCM-199 containing 5 mM caffeine for 5 min, there were variations in the sperm concentration at the places in the exit chamber of MFSS. In the second experiment, when porcine IVM oocytes were placed at three different places in the exit chamber of MFSS, where motile spermatozoa will accumulate with mM199 containing caffeine, for 5 min and then cultured in caffeine-free mM199 for 8 h, sperm penetration rate was not different among the places, but monospermic penetration rate was. In the last experiment, we compared sperm penetration in this MFSS-IVF system with a standard IVF method (co-cultured in droplets for 8 h). The normal fertilization index (the ratio of monospermic oocytes to the number of oocytes examined) 8 h following insemination was higher in the MFSS-IVF system than the standard IVF system. Developmental competence of fertilized oocytes to the blastocyst stage was also higher in the MFSS-IVF system than standard IVF. These results demonstrate that a short co-culture of porcine oocytes with spermatozoa gradually accumulated in the MFSS chamber improves the efficiencies of normally fertilized embryo production and blastocyst formation, whereas these efficiencies may be affected by the place where oocytes are located in the chamber.

Introduction

A high incidence of polyspermic penetration is a big obstacle to obtain normal zygotes following in vitro fertilization (IVF) of porcine oocytes matured in vitro (IVM) [1-4]. Simultaneous sperm penetration and delay of zona reaction at the first sperm penetration may be a large reason to induce polyspermic penetration in vitro [1,5]. In porcine standard IVF, generally thirty to fifty denuded or cumulus-enclosed oocytes are co-cultured with 1-10 x 10^4 spermatozoa in a droplet of IVF medium containing caffeine for several hours. Since caffeine, a cyclic-nucleotide phosphodiesterase inhibitor, increases the intracellular cyclic AMP concentration [6] and induces sperm capacitation and the spontaneous acrosome reaction [7], a number of spermatozoa induced capacitation simultaneously try to penetrate into the oocytes. Consequently, the incidence of polyspermic penetration increases significantly if caffeine is supplemented to IVF media [8-10]. In the porcine oviducts, on the other hand, since spermatozoa being reserved at the utero-tubal junction are induced the capacitation and start to enter the site of fertilization, oocytes should meet the gradual number of capacitated spermatozoa. According to the above idea, unique IVF systems, such as climbing-over-the wall method[11], straw-IVF method[12,13], have been developed. Due to the limited improvements in these methods, however, further development of a new simple IVF method will be required for the supply of normal embryos in vitro stably and efficiently.

Furthermore, centrifugation has been utilized to wash and prepare spermatozoa before insemination. However, centrifugation appears to injure the quality of spermatozoa in rodents [14,15] and human [16]. Recently, micro-channel has been utilized as a new tool for biological research and applied for artificial reproductive technologies during IVM, IVF and early development of mammalian oocytes [17]. To wash and isolate motile spermatozoa without

centrifugation, a simple micro-channel device, microfluidic sperm sorter (MFSS), has been developed by using microfluidics and laminar flow [18,19]. In this device, two parallel laminar flows stream in a micro-channel and then separate to each different direction. Only motile spermatozoa can swim across contacting surface area of laminar flows, deviate from the initial streamline into the media stream for collection, and gradually accumulate in the collection chamber. If porcine oocytes can be co-cultured with motile spermatozoa in the chamber of MFSS, the gradual accumulation of motile and uninjured spermatozoa can be induced in the meeting place with oocytes without centrifugation, and consequently may improve the efficiency to obtain monospermic embryos.

In the present study, we examined if the application of the microfluidic sperm sorter to co-culture of porcine oocytes with isolated motile spermatozoa improved the efficiency to produce normal fertilized embryos.

Materials and methods

Chemicals and Culture Media

Potassium chloride, KH_2PO_4, $MgCl_2 \cdot 6H_2O$, $CaCl_2 \cdot 2H_2O$, sodium citrate and citric acid were purchased from Ishizu Pharmaceutical Co., Ltd (Osaka, Japan). Sodium chloride and paraffin liquid were obtained from Nacalai Tesque Inc. (Kyoto, Japan). Unless specified, other chemicals were purchased from Sigma Aldrich Japan K.K. (Tokyo, Japan).

The medium used for collecting and washing COC was modified TL-HEPES-PVA medium composed of 114 mM NaCl, 3.2 mM KCl, 2 mM $NaHCO_3$, 0.34 mM KH_2PO_4, 10 mM Na-lactate, 0.5 mM $MgCl_2 \cdot 6H_2O$, 2 mM $CaCl_2 \cdot 2H_2O$, 10 mM HEPES, 0.2 mM Na-pyruvate, 12 mM sorbitol, 0.1% (w/v) polyvinylalcohol, 25 μg/ml gentamicin and 65 μg/ml potassium penicillin G.

The basic IVM medium used was a BSA-free chemically defined medium, POM (Research Institute for the Functional Peptides, Yamagata, Japan) supplemented with 50 μM beta-mercaptoethanol (mPOM). This IVM medium supports blastocyst development well following IVF if gonadotropins are added during IVM [20]. Medium for IVF was Medium-199 that was supplemented with 3.05 mM glucose, 2.92 mM hemi-Ca lactate, 0.91 mM sodium pyruvate, 12.0 mM sorbitol, 75mg/l penicillin G potassium, 25 mg/l gentamicin and 4 mg/ml (w/v) BSA (mM-199). Medium for in vitro development to the blastocyst stage was PZM-5 (Research Institute for the Functional Peptides, Yamagata, Japan). All media (except modified TL-HEPES-PVA) were equilibrated at 39 °C in an atmosphere of 5% CO_2 in air overnight prior to use.

Preparation and culture of cumulus-oocyte complexes

Ovaries were collected from slaughtered prepubertal gilts at a local abattoir and transported to the laboratory in 0.9% NaCl containing 75 μg/ml potassium penicillin G and 50 μg/ml streptomycin sulphate. Using an 18-gauge needle and a disposable 10-ml syringe, COC were aspirated from antral follicles (3 to 6 mm in diameter) on the surface of ovaries and washed three times with modified TL-HEPES-PVA medium at chamber temperature (25 °C) [21]. Forty to fifty COC with uniform ooplasm and a compact cumulus cell mass were washed three times with IVM medium. These complexes were subsequently cultured in 500 μl of IVM medium added 1 mM dibutyryl cAMP, 10 IU/ml eCG and 10 IU/ml hCG for 20 h, and then in 500 μl of IVM medium without additives for a further 24 h period, at 39 °C in an atmosphere of 5% CO_2 in air. After IVM culture, oocytes were stripped of cumulus cells by pipetting with 0.1% (w/v) hyaluronidase.

Preparation of fresh boar spermatozoa

Semen-rich fractions (about 50 ml) were collected from totally four Birkshire boars by glove-hand method at a local experimental station and were diluted 5 times with modified Modena solution [22]. The diluted semen samples were transported to the laboratory within 2 h of collection at 26-32 ^{o}C. Spermatozoa was diluted at a concentration of 1 x 10^8 cells/ml with modified Modena solution containing 5 mM cysteine and 20% (v/v) boar seminal plasma. This diluted semen was directly used for insemination in the MFSS. For standard IVF, ten milliliters of the re-suspended spermatozoa were washed three times by centrifugation at 750 *g* for 3 min with modified TL-HEPES-PVA solution and then re-suspended at a concentration of 1 x 10^8 cells/ml in mM199.

IVF in the MFSS

The micro-channel in the MFSS (STREX Inc., Osaka, Japan) was filled up with 5 μl of mM199 containing 5 mM caffeine-benzoate, two hundred micro-liter each of the same medium was added to the chambers D and C, and then 800 μl of the same medium was added to the chamber B (Figure 1). Denuded oocytes were placed at the b, c or d point in the chamber D (Figure 2), and then flowing was started by adding 550 μl of diluted fresh semen to the chamber A. After flowing for 5 min at room temperature, the denuded oocytes attaching spermatozoa in the chamber D were gently transferred to 500 μl of caffeine-free mM199 by using a relatively big boa size of Pasteur pipette and then cultured at an atmosphere of 5% CO_2 in air at 39 ^{o}C for totally 8 h following insemination.

Standard IVF

After dilution to 11.4 x 10^5 cells/ml with mM199, fifty micro-liter of diluted sperm

suspension was inseminated in the same volume of mM199 containing 10 mM caffeine-benzoate (final sperm concentration was 5.7 x 10^5 cells/ml). Thirty to forty denuded oocytes were co-cultured with spermatozoa in 100 μl droplet of mM199 containing finally 5 mM caffeine-benzoate for 8 h at 39 °C in an atmosphere of 5% CO_2 in air.

Assessment of sperm penetration and early development

Eight hours after insemination, some of the cultured eggs were fixed, stained with 1% (w/v) orcein, and examined at 200x and 400x magnification. Oocytes were designated as penetrated when they had at least one sperm head, a decondensed sperm nucleus, or a male pronucleus and corresponding sperm tail in the vitellus. Other oocytes were incubated in 500 μL of PZM-5 for 7 days at 39 °C in an atmosphere of 5% CO_2 in air. Cleavage and morula/blastocyst formation of the oocytes were examined on 2 and 7 days after the start of culture, respectively.

Experimental design.

In the first experiment, five minutes after the start of flowing, five micro-liter of media at the positions a, b, c and d in the chamber D were collected, and then sperm concentration of each medium was determined by using a hemocytometer (Table 1).

In the second experiment, eight to ten denuded oocytes were placed at the positions b, c and d in the chamber D, exposed to flowed spermatozoa for 5 min and then cultured in a caffeine-free fresh medium for totally 8 h. Sperm penetration of the oocytes was assessed (Table 2).

In the last experiment, thirty denuded oocytes were placed at the position c in the chamber D, exposed to flowed spermatozoa for 5 min and then cultured in a caffeine-free fresh medium for totally 8 h. Sperm penetration of the oocytes was compared with that of oocytes co-cultured

with spermatozoa for 8 h in a standard drop IVF method (Table 3). Furthermore, early developmental competence of the oocytes fertilized in MFSS-IVF syatem was also examined by incubating in PZM-5 at 39 °C in an atmosphere of 5% CO_2 in air for 7 days, and then compared with that of oocytes fertilized in a standard droplet IVF system (Table 4).

Statistical analysis.

When the percentage data were contained less than 10% and more than 90%, all percentage data were subjected to arc sine transformation before statistical analysis. Statistical analyses of results from 5 replicates were used for treatment comparisons and carried out by analysis of variance (ANOVA) using the STATVIEW (Abacuc Concept, Inc., Berkeley, CA, USA) program. If the P value was smaller than 0.05 in ANOVA, Tukey-Kramer's HSD test was followed using the same program. All data were expressed as means ± SEM. $P \leq 0.05$ was considered to be statistically significant.

Results

In the first experiment, there were large differences in sperm concentration among positions a, b, c and d in the chamber D following flowing for 5 min (Figure 1). At the position a, around the exit from micro-channel, a huge number of spermatozoa accumulated as compared with other positions. Furthermore, sperm concentration at the position b was also significantly higher than positions c and d. There was not a difference in sperm concentration between positions c and d.

In the second experiment, eight hours after insemination, there were no differences in sperm penetration rates among places where denuded oocytes were placed at, exposed to

flowed spermatozoa for 5 min (Table 2). However, both the incidence of monospermic penetration and normal fertilization index (the ratio of monospermic oocytes to the number of oocytes examined) were significantly higher when denuded oocytes were placed at the positions c and d than b in the chamber D. However, there were no differences in the monospermy rate and the normal fertilization index when the oocytes were placed at the positions c and d.

In the last experiment, when thirty denuded oocytes were placed the position c in the chamber D of MFSS, exposed to flowed spermatozoa for 5 min and then cultured in a caffeine-free fresh medium for totally 8 h, the penetration rate was not significantly different with that of oocytes co-cultured for 8 h in a standard drop IVF method. However, both the incidence of monospermic penetration and the normal fertilization index were significantly higher when denuded oocytes were placed at the position c in the chamber D of MFSS, as compared with a standard drop IVF method (Table 3). Furthermore, early developmental competence of oocytes to the blastocyst stage was also higher when the oocytes were inseminated in the MFSS device as compared with those inseminated in a standard droplet IVF system (Table 4).

Discussion

In order to isolate motile human spermatozoa for artificial reproductive technology without centrifugation, recently, MFSS has been developed [19]. In this device, motile spermatozoa can gradually accumulate in an exit chamber. We examined if porcine oocytes co-cultured with gradually accumulated motile spermatozoa in the chamber of this microfluidic sperm sorter could improve the efficiency to produce normally fertilized zygotes. In IVF of porcine IVM

oocytes, a long co-culture time appears to increase both the incidences of sperm penetration and polyspermic penetration [23-27]. A transient co-incubation of oocytes with spermatozoa has reported to increase the incidence of normal fertilized zygotes [13,27,28]. Application of a MFSS device utilized microfluidics and laminar flow to the transient co-culture IVF system would extract only motile spermatozoa [19] and efficiently remove dead spermatozoa, which affect detrimentally sperm-egg fusion [29]. In this centrifugation-free MFSS-IVF system, furthermore, injured spermatozoa induced by centrifugation [14-16] was also not included. In the present study, transient exposure of oocytes to isolated spermatozoa in the MFSS produced successfully fertilized oocytes. Even with a relatively few number of spermatozoa at the positions b and c in the chamber D of MFSS (3.3×10^4 and 1.5×10^4 cells/ml, respectively) and very short exposure time (for 5 min), very stably high sperm penetration was achieved (100% and 97.2 $\pm$ 2.8 %) in this MFSS-IVF system. Suh et al. [30] have also demonstrated that murine IVF within their original microfluidic channel IVF device was conducted successfully with lower total numbers and concentrations of spermatozoa. Our result was consistent with this previous result. These results suggest that removing dead spermatozoa and isolation of uninjured motile ones should improve the efficiency of sperm-egg binding and consequent sperm penetration.

In the current study, we found that polyspermic penetration rates varied at the positions b, c and d in the chamber D of MFSS. The distance from the exit of micro-channel was about 3.5, 7.5 and 8.3 mm, respectively. The number of sperm distribution and the normal fertilization index (the ratio of monospermic oocytes to the number of oocytes examined) appear to be reflected directly and inversely by the distance from the exit of micro-channel. In these positions, not only the number of spermatozoa accumulated around the oocytes but also the speed of the accumulation was different. These factors may also affect the index of normal

fertilization in this MFSS-IVF system. Furthermore, when MFSS-IVF at the position c in the chamber D was compared with standard IVF system, both the incidence of monospermic penetration and the normal fertilization index in MFSS-IVF was much higher than that in standard IVF. These evidences suggest that selection of motile spermatozoa and gradual accumulation of the spermatozoa around oocytes make possible to penetrate with a small number of spermatozoa, reduce the incidence of simultaneous sperm penetration, and consequently has advantage to achieve normal fertilization in vitro in porcine IVM oocytes.

We also observed that the developmental competence of oocytes fertilized in the MFSS-IVF system, as assessed by in-vitro development to the blastocyst stage, was almost twice higher than that of oocytes fertilized in a standard droplet IVF system. This shows that MFSS-IVF has an advantage in the efficiency to produce porcine blastocysts in vitro. In the current study, furthermore, difference in the developmental competence reflected well that in the normal fertilization index. Polyspermic penetration is believed to result in the developmental failure of the zygotes [31], with a reduced blastocyst formation and the quality [32], whereas Han et al. [33] demonstrated that polyspermic IVM-IVF porcine embryos containing multiple pronuclei developed in vitro to the blastocyst stage at the same percentage as monospermic IVM-IVF embryos. Results in the current study was support the former observations.

In the present study, when eight to ten denuded oocytes were placed at the position c of the MFSS chamber D, the normal fertilization index was 0.521 $\pm$ 0.069 (Table 2), whereas it decreased to 0.222 $\pm$ 0.028 (Table 3) when thirty denuded oocytes were placed. This shows that the number of oocytes placed in the chamber is also important factor to affect the normal fertilization index. In a standard IVF system inseminated in droplets containing caffeine, a high correlation between the incidence of polyspermy and the number of spermatozoa per oocyte in

the fertilization medium [34]. In the MFSS-IVF system also, the number of oocytes placed and/or the concentration of spermatozoa sorted should be regulated as obtaining a high score of the normal fertilization index.

In conclusion, application of MFSS, which permits gradual accumulation of only motile spermatozoa without centrifugation by using microfluidics and laminar flow, to a transient co-culture IVF system increased significantly the incidence of normal fertilization without any significant reductions in the fertilization rate, and consequently the efficiency of the embryos to develop to the blastocyst stage. However, these efficiencies appear to be affected by the place where oocytes are located in the chamber and the number of oocytes.

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Legend of figure

Figure 1. Photograghs of mircofluidic sperm sorter. The upper figure shows the side of the chamber. Note a difference in the thickness of wings between left and right sides (arrows). The middle figure shows micro-channels among four chambers. The lower figure shows a micrograph of the device where the micro-channels deviate (it is the same place where a white arrow shows in the middle figure). Micro-streams of semen and medium flow together side-by-side in a laminar fashion and exit to the different chambers. Only motile spermatozoa can swim across the laminar flows and can be isolated in the medium downstream.

Figure 2. In the chamber D of mircofluidic sperm sorter, the places where sperm concentration was examined (a-d) and where oocytes were placed in IVF experiment (b-d). The “a” area was the exit of micro-channel to the chamber D. The “b”, “c” and “d” areas were the vertexes of rectangle with 3.5 mm x 7.5 mm in the chamber D. The bar in the upper right corner shows 3 mm.

Table 1. Sperm concentration at various areas in the chamber D of mircofluidic sperm sorter following sperm flowing for 5 min

Observation Area[1]	Sperm concentration (x 10^4 cells/ml)
a	575.0 ± 56.3[a]
b	3.3 ± 0.8[b]
c	1.5 ± 0.6[c]
d	0.8 ± 0.5[c]

[1] Five micro-liters of sperm sample was collected at each position in the chamber D of a microfluidic sperm sorter (see figure 2).

Data are given as mean ± SEM from five replicated experiments. Values with different superscripts within column are significantly different ($P < 0.05$).

Table 2. Effect of the position of oocytes in the chamber D of a mircofluidic sperm sorter on sperm penetration in vitro following sperm flowing for 5 min

Positions where oocytes were placed [1]	No. of oocytes examined	No. (%)[2] of oocytes penetrated	No (%)[3] of monospermic oocytes	Normal fertilization index[4]
b	38	38 (100)	5 (12.5 ± 4.8)[a]	0.125 ± 0.048[a]
c	33	32 (97.2 ± 2.8)	17 (53.1 ± 6.0)[b]	0.521 ± 0.069[b]
d	38	33 (86.1 ± 10.5)	14 (41.9 ± 2.8)[b]	0.364 ± 0.058[b]

[1] Denuded oocytes (8-10 oocytes per replication) were placed at each position (b-d) of the chamber D in a Microfluidic sperm sorter (see figure 2), exposed to flowed spermatozoa for 5 min and then cultured in the caffeine-free media for 8 h.

[2] Percentage based on the total number of oocytes examined.

[3] Percentage based on the number of oocytes penetrated.

[4] Ratio of monospermic oocytes to the number of oocytes examined

Data are given as mean ± SEM from five replicated experiments.

Values with different superscripts within column are significantly different ($P < 0.05$).

Table 3. Comparison of sperm penetration in microfluidic sperm sorter following sperm flowing for 5 min with other standard IVF systems

IVF Methods[1]	No. of oocytes examined	No. (%)[2] of oocytes penetrated	No (%)[3] of monospermic oocytes	Normal fertilization index[4]
Standard	109	96 (87.9 ± 8.7)	24 (27.1 ± 6.8)[a]	0.222 ± 0.028[a]
MFSS-IVF	105	65 (59.0 ± 11.4)	40 (68.6 ± 10.5)[b]	0.375 ± 0.040[b]

[1]Standard: thirty to forty denuded oocytes were placed in a droplet and co-cultured with 5.7 x 10^5 cells/ml spermatozoa for 5 min or 8 h. MFSS: thirty to forty denuded oocytes were placed at the position c in the chamber D of a Microfluidic sperm sorter and semen were flowed for 5 min. The oocytes co-cultured for 5 min in droplet or flowed for 5 min in MFSS were continued culture in caffeine-free medium for another 8 h.

[2]Percentage based on the total number of oocytes examined.

[3]Percentage based on the number of oocytes penetrated.

[4] Ratio of monospermic oocytes to the number of oocytes examined

Data are given as mean ± SEM from four replicated experiments.

Values with different superscripts within column are significantly different ($P < 0.05$).

Table 4. Comparison of early development following IVF in microfluidic sperm sorter with those after IVF in other standard IVF systems

IVF Methods[1]	No. of IVF oocytes examined	No (%)[2] of IVF oocytes developed to > 2-cell stage	No (%)[2] of IVF oocytes developed to the blastocyst stage
Standard	126	104 (80.5 ± 8.0)	28 (22.6 ± 1.4)[a]
MFSS-IVF	123	109 (88.3 ± 2.2)	51 (40.9 ± 2.3)[b]

[1]Standard: thirty to forty denuded oocytes were co-cultured with 5.7 x 10^5 cells/ml spermatozoa in a droplet for 5 min or 8 h. MFSS: thirty to forty denuded oocytes were placed at the position c in the chamber D of a Microfluidic sperm sorter and semen were flowed for 5 min. The oocytes co-cultured for 5 min in droplet or flowed for 5 min in MFSS were continued culture in caffeine-free medium for another 8 h. Those IVF oocytes were cultured in PZM-5 for 7 days.

[2]Percentage based on the total number of oocytes examined.

Data are given as mean ± SEM from four replicated experiments.

Values with different superscripts within column are significantly different ($P < 0.05$).