IN VITRO FERTILIZATION OF PORCINE OOCYTES: COMPARISON OF FERTILITY IN AI - BOARS

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Abstract

The aim of the study was to compare *in vitro* fertility in boars with high field fertility to select those suitable for embryo production and determine conditions for oocyte fertilization in terms of spermatozoa treatment and time of gamete coincubation. Fresh semen of four AI-boars, A, B, C and D were used for the experiments. Oocytes were matured by standard protocol. They were fertilized by spermatozoa isolated by Percoll gradient and preincubated in modified Tris-buffered medium (mTBM) without or with 1.5 mM caffeine for 30 min. Both the untreated and caffeine treated spermatozoa were coincubated with oocytes for 3h, 3h 45min or 4h 30min. Oocytes were cultured in PZM-3 medium for twelve hours and than fixed, stained with 33258-Hoechst and examined by epifluorescence. The penetration rates were higher for the untreated spermatozoa of boars A and B compared with those of boars C and D (74.1 ± 11.2 and 77.3 ± 2.8% vs. 12.1 ± 2.8 and 19.7 ± 0.9%). The penetration rate increased from 19.7 ± 0.9 to 74.4 ± 11.4% in spermatozoa of boar D after the caffeine treatment but not in boar C. The normal fertilization rates for untreated spermatozoa of boars A and B were higher than those of boars C and D (25.9 ± 4.2% and 20.6 ± 5.2 vs. 3.0 ± 0.3 and 6.1 ± 0.2%). They increased to 28.8 ± 4.6% in boar B but decreased to 15.4 ± 1.5% in boar A after the spermatozoa treatment; no response was found in boars C and D. In order to improve the course of fertilization process, gamete coincubation time was modified for IVF-suitable boars. The 4h and 30min-coincubation of oocytes with either untreated or caffeine treated spermatozoa was more effective in boars A and B respectively compared with 3h-coincubation interval. It can be concluded that a) It is important to test in vitro fertility of AI-boars before their employment for embryo production b) There is variability in response of boar spermatozoa to caffeine treatment during fertilization c) Independently on the spermatozoa response, the efficiency of fertilization is influenced by time of gamete coincubation.

Introduction

Low efficiency of monospermic fertilization and high incidence of polyspermy is a major problem during *in vitro* fertilization of porcine oocytes and embryo production. There is relatively much information as concerns various factors influencing the fertilization process but some of them are contradictious. First of all, experiments were done to modify fertilization conditions, such as medium composition, spermatozoa treatment and their concentration, sperm oocyte ratio or time of gamete interaction but no important improvement was achieved up to now (Abeydeera and Day, 1996; Behalova *et al*., 1993; Gil *et al*., 2007; Coy *et al*., 1993; Funahashi and Romar, 2004). Large variations among individual boars have been described in efficiency of fertilization with fresh and frozen sperm (Sirard *et al*., 1993; Suzuki *et al*., 2005), however the variations in fertilization and polyspermy in individual boars are smaller than those in different breeds (Suzuki *et al*., 2003). Only little information is available for predicting *in vitro* fertilizing ability of spermatozoa in order to optimize the fertilization process in various boars and eliminate those which are inappropriate for in vitro fertilization and production of embryos (Gadea *et al,*. 1998).

Our study was designed to compare *in vitro* fertility of AI-boars with known field fertility, to eliminate those which are not suitable for *in vitro* system, and estimate conditions for oocyte fertilization in IVF-suitable boars in terms of spermatozoa treatment and time of gamete coincubation.

Materials and methods

Semen donors

Semen of four AI-boars, A, B, C and D, Landrace breed, with high field fertility was tested in our experiments. Fresh, BTS extended, semen was used within eight hours after collection.

Oocyte donors

Ovaries from adult cyclic sows were obtained at local abattoir and transported to the laboratory within 2 h at 31 - 33°C. Ovaries were examined for their morphology to determine donor estrous cycle status and only those from the middle luteal to the early follicular phase were used for oocyte collection. Oocytes were isolated by slicing of ovarian cortex.

Oocyte maturation

Only healthy oocyte-cumulus complexes with a dark, evenly granulated cytoplasm and at least two compact layers of cumulus cells were used for maturation. COCs in group of 30 - 35 were matured in 500 mL of TCM-199 medium (Earle′s salt), with addition of 0.20 mM sodium pyruvate, 0.57 mM cysteamin, 50 IU·mL^{-1} penicillin, 50 mg·mL^{-1} streptomycin (Sigma Chemicals Co., Prague, Czech Republic), 10 % FCS (fetal calf serum), and gonadotropins (P.G.600 15 IU·mL^{-1}; Intervet, Boxmeer, Holland) in a Nunc 4-well multi-dish (Nunc, Intermed, Roskilde, Denmark) for 46-47 h at 39°C in a humidified

Spermatozoa treatment
Semen of every boar was washed by centrifugation at 220×g for 10min in Dulbecco's phosphate buffered saline (Dulbecco - PBS) supplemented with 0.01% polyvinylalcohol (PVA). The sperm was layered on 40 vs. 80% Percoll density gradient (GE Healthcare Bio - Sciences AB, Uppsala, Sweden) and motile spermatozoa were isolated by centrifugation at 560×g for 20min. Pellet was washed twice by centrifugation at 220×g for 10min in Dulbecco - PBS – PVA and resuspended in fertilization medium described by Abeydeera and Day (1997) as modified Tris-buffered medium (mTBM) containig 113.1 mM NaCl, 3 mM KCl, 10 mM $CaCl_2.H_2O$, 20mM Tris, 11mM glucose, 5mM sodium pyruvate and supplemented with 0.4% BSA. Spermatozoa were adjusted to concentration 1×10^6 and than were preincubated in 500ml of mTBM medium without or with 1.5 mM caffeine for 30 min at 39 °C in atmosphere with 5 % CO_2.

Spermatozoa oocyte coincubation
Both untreated and caffeine treated spermatozoa were added to oocytes denuded by vortex in the ratio 1250 of spermatozoa per oocyte. They were coincubated with oocytes in mTBM medium for 3h 45min at 39°C in atmosphere with 5% CO_2. In order to improve the penetration and fertilization for IVF-suitable boars, the coincubation time was modified to both 3h, and 4h 30min for boars A and B. To remove spermatozoa attached to zona pellucida, the oocytes were vortexed after coincubation.

Oocyte culture and examination
Oocytes were cultured in PZM-3 medium (Yoshioka *et al*., 2002) for twelve hours, than fixed in 2.5 % aqueous glutaraldehyde solution (v/v), stained with bisbenzimide-33258 Hoechst in citrate buffer (0.154 $mol\cdot L^{-1}$ sodium chloride and 0.015 $mol\cdot L^{-1}$ trisodium citrate) at room temperature for 10 minutes, and rinsed three times in Dulbecco-PBS. Wet mounts were prepared in 5 mL glycerin buffer and the oocytes were examined by epifluorescence at a magnification × 400.

Fertilization assessment
Oocytes were determined to be penetrated when one or more swollen sperm head(s) and/or male pronucleus (pronuclei) were found. Oocytes with more than one swollen sperm head or male pronucleus were assessed as polyspermic. Those with either one swollen head and female pronucleus or one male pronucleus and metaphase II were determined as asynchronic. Only oocytes with one male and one female pronucleus and two polar bodies were determined as monospermic.

Data analysis
The data were expressed as mean percentages ± S.D. of penetrated oocytes from inseminated oocytes, and polyspermic, asynchronic or monospermic oocytes from penetrated oocytes. Efficiency of normal fertilization was assessed as mean percentage ± S.D. of monospermic oocytes from inseminated oocytes.

Results

The efficiency of penetration and fertilization of oocytes with spermatozoa of the tested boars related to the caffeine treatment is shown in Table 1. The penetration rates were higher for the untreated spermatozoa of boars A and B compared with those of boars C and D. The penetration rate increased in spermatozoa of boar D after the caffeine treatment but not in boar C. The polyspermy rates were lower for untreated spermatozoa of boars A, B and D in comparison with those of boar C; polyspermy increased in boars A and D but decreased in boars B and C after the spermatozoa treatment. The normal fertilization rates for untreated spermatozoa of boars A and B were higher than those of boars C and D. The normal fertilization rate increased in boar B but decreased in boar A after the spermatozoa treatment. Concerning this parameter, no response to caffeine was found in boars C and D after spermatozoa treatment.

Table 1. Efficiency of oocyte fertilization related to spermatozoa treatment (means ± S.D.)

Boar	Sperm treatment by caffeine	Oocytes inseminated	Oocytes penetrated	Oocytes penetrated			Efficiency of normal fertilization
				polyspermic	asynchronic	monospermic	
		(n)	*(%)*	*(%)*	*(%)*	*(%)*	*(%)*
A	-	108	74.1 ± 11.2	61.2 ± 3.1	3.8 ± 3.9	35.0 ± 1.1	25.9 ± 4.2
	+	175	76.6 ± 10.8	76.2 ± 5.1	3.7 ± 2.5	20.1± 2.4	15.4 ± 1.5
B	-	97	77.3 ± 2.8	66.7 ± 10.1	6.7 ± 4.9	26.6 ± 5.8	20.6 ± 5.2
	+	198	71.7 ± 8.1	56.3 ± 10.1	3.5 ± 3.7	40.2 ± 6.7	28.8 ± 4.6
C	-	66	12.1 ± 2.8	75.0 ± 0	0	25.0 ± 0	3.0 ± 0.3
	+	205	15.6 ± 3.3	50.0 ± 34.7	28.1± 36.5	21.9 ± 15.6	3.4 ± 2.1
D	-	66	19.7 ± 0.9	61.6 ± 4.8	7.6 ± 7.2	30.8 ± 2.4	6.1± 0.2
	+	207	74.4 ± 11.4	91.6 ± 4.4	2.6 ± 3.5	5.8 ± 3.1	4.3 ± 1.5

coincubation time : 3h 45min

According to fertilization characteristics, the sires were categorized as boars with or without response to caffeine and suitable or not suitable for in vitro fertilization (Table 2). Only spermatozoa of boars D and B responded to caffeine treatment, in terms of increase in penetration and normal fertilization respectively, but no response of spermatozoa was found in boars A and C. Without regard to spermatozoa response to caffeine, only boars A and B were suitable for IVF because in these sires, higher efficiency of penetration and normal fertilization was achieved in comparison with boars C and D.

In order to improve the course of fertilization process, gamete coincubation time was modified for IVF-suitable boars. The efficiency of oocyte penetration and fertilization with spermatozoa of boars A and B related to time of coincubation is shown in Table 3. The 4h and 30min-coincubation of oocytes with either untreated or caffeine treated spermatozoa seemed to be more effective for boars A and B respectively compared with 3h-coincubation interval.

Table 2. Characteristics of oocyte penetration and fertilization by spermatozoa of the tested boars

Boar	Sperm respons to caffein	Rates			Suitable for IVF
		penetration	polyspermy	monospermy	
A	no	high	medium	high	yes
B	yes	high	medium	high	yes
C	no	low	high	low	no
D	yes	high	high	low	no

Table 3. Efficiency of oocyte fertilization related to time of coincubation (means ± S.D.)

Boar	Coincubation time	Sperm treatment by caffein	Oocytes inseminated	Oocytes penetrated	Oocytes penetrated			Efficiency of normal fertilization
					polyspermic	asynchronic	monospermic	
			(n)	*(%)*	*(%)*	*(%)*	*(%)*	*(%)*
A	4h 30min	-	108	74.1 ± 11.2	61.2 ± 3.1	3.8 ± 3.9	35.0 ± 1.1	25.9 ± 4.2
	3h	+	107	69.2 ± 5.2	73.0 ± 3.7	5.4 ± 1.9	21.6 ± 2.0	15.0 ± 1.5
	4h 30min	+	68	88.2 ± 5.5	80.0 ± 4.0	1.7 ± 1.7	18.3 ± 2.3	16.1 ± 1.0
B	4h 30min	-	97	77.3 ± 2.8	66.7 ± 10.1	6.7 ± 4.9	26.6 ± 5.8	20.6 ± 5.2
	3h	+	99	66.7 ± 5.2	53.0 ± 8.6	4.5 ± 3.4	42.5 ± 5.6	28.3 ± 4.1
	4h 30min	+	99	76.8 ± 7.4	59.2 ± 10.9	2.6 ± 3.8	38.2 ± 7.4	29.3 ± 5.0

Conclusion

It can be concluded that a) It is important to verify *in vitro* fertility in boars with high field fertility before their use for embryo production b) There is variability in response of boar spermatozoa to caffeine treatment during fertilization c) Independently on the spermatozoa response, the efficiency of fertilization is influenced by time of gamete coincubation.

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