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In vitro production of bovine embryos using flow-cytometrically sexed sperm*

Abstract

The investigation aimed to compare the effect of fresh and frozen-thawed X and Y fractions of flow-cytometrically sorted bovine spermatozoa on in vitro fertilization of bovine in vitro matured oocytes and subsequent blastocyst development. Sperm cells sorted in MoFloSX cytometer were used either for IVF or frozen and stored in liquid nitrogen. Immature oocytes recovered from ovaries of slaughtered animals and matured in vitro in TCM-199 containing 20% estrus cow serum and additional granulosa cells were fertilized in vitro with fresh or frozen-thawed fractions of sorted sperm. Simultaneously, control, fresh or frozen/thawed sperm was used for IVF. A total number of 2712 IVM oocytes were fertilized with sorted and control sperm of 6 bulls. Embryo cleavage rates were significantly affected by bull ($P<0.0001$), sperm sexing ($P<0.0001$) and sperm freezing ($P<0.01$). Blastocysts development was affected by sperm freezing ($P<0.04$) and sperm sexing ($P<0.01$). The significant differences were shown between unsorted and sorted sperm, however no differences in embryo cleavage rates and blastocysts rates were observed between X- and Y-sperm fractions, both fresh and frozen/thawed. There were significant differences in cleavage rates among fresh, control sperm (52.7%), X fraction (26.8%) and Y fraction (24.7%). Similar differences in cleavage rates were shown for frozen/thawed control sperm (52.8%), X fraction (33.9%) and Y fraction (26.2%). The female blastocysts were frozen for further transfer, while the hatched male blastocysts were analysed by PCR revealing 76.2% accuracy.

The results suggest that there were significant differences in cleavage rates and blastocyst rates due to sperm sorting in comparison to unsorted sperm and no differences between effectiveness of X and Y fractions of spermatozoa.

Key Words: cattle, sperm sexing, flow-cytometry, IVM, IVF, blastocyst

Zusammenfassung

Titel der Arbeit: **In vitro Produktion von Rinderembryonen nach Verwendung von geschlechtsdeterminierten Spermien**

Die Untersuchungen wurden mit dem Ziel durchgeführt, den Effekt von frischen und eingefrorenen/aufgetauten X- und Y-tragenden Spermienfraktionen nach der Sortierung im Durchflusszytometer auf die In vitro-Fertilisation und die Blastozystenentwicklung von in vitro gereiften Rinderoozyten zu vergleichen.

Die im MoFloSX-Zytometer geschlechtsseparierten Spermien wurden entweder für die IVF verwendet oder eingefroren und in flüssigem Stickstoff gelagert. Unreife Oozyten aus Schlachthausovarien wurden in vitro in TCM-199 + 20% östrischem Kuhserum und zusätzlichen Granulosazellen gereift. Die IVF erfolgte mit frischen oder tiergefrorenen/aufgetauten Fraktionen der sortierten Spermien. Insgesamt wurden 2712 Ooztyten nach IVM mit Spermien von 6 verschiedenen Bullen befruchtet. Die Teilungsrate der Embryonen war signifikant beeinflusst durch den Bullen ($P<0.0001$), die Spermienseparierung ($P<0.0001$) und die Kryokonservierung ($P<0.01$). Die Blastozystenentwicklung wurde durch die Kryokonservierung der Spermien ($P<0.04$) und die Spermienseparierung ($P<0.01$) beeinflusst. Ein signifikanter Unterschied wurde auch zwischen unseparierten und separierten Spermien aufgezeigt, obwohl keine Unterschiede in der Teilungsrate der Embryonen und der Blastopzystenrate zwischen den X- und Y-fraktionierten Spermien sowohl bei frischen als auch bei eingefrorenen Spermien beobachtet wurden. Signifikante Unterschiede in den Furchungsraten konnten zwischen der Kontrollen mit Frischsperma (52.7%) und den X- (26.8%) und Y-Fraktionen (24.7%) beobachtet werden. Ähnliche Unterschiede wurden auch bei Verwendung von tiefgefrorenen/aufgetauten Kontrollspermien (52.8%) und den beiden Fraktionen (X – 33.9.5%; Y – 26.2%) erzielt. Die als weiblich determinierten Blastozysten wurden für einen späteren Transfer eingefroren, während die als männlich determinierten geschlüpften Blastozysten der PCR zugeführt wurden. Es konnte eine Genauigkeit im Geschlecht von 76.2% erzielt werden.

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Die Ergebnisse zeigen, dass signifikante Unterschiede in den Furchungs- und Blastozystenraten zwischen geschlechtsdeterminierten und nichtdeterminierten Spermien bestanden, jedoch keine Unterschiede zwischen den X- und Y-Fraktionen vorhanden waren.

Schlüsselwörter: Rind, Geschlechtsdeterminierung, Durchflusszytometrie, IVM, IVF, Blastozystenrate

Introduction

Gender preselection in mammals may be carried out both by segregation of spermatozoa or by transfer of embryos with predetermined sex. However, embryo sexing is not only expensive but may affect developmental competence as well because of damage of embryo integrity. Therefore, sperm sexing and separation into X- and Y-bearing fractions for purposes of insemination or IVF seems to be a distinctly more useful procedure in gender predetermination of mammals. The only successful method of sexing spermatozoa is quantifying sperm DNA-binding dye, followed by flow cytometry and cell sorting.

The previous experiments on bovine IVF (MILLER and HUNTER, 1987; AOYAGI et al., 1988; EYESTONE and FIRST, 1989; KĄTSKA et al., 1996; CHAMBERLAND et al., 2001; KĄTSKA-KSIĄŻKIEWICZ et al., 2005) have shown that in terms of factors affecting the outcome of the procedure bull variability has appeared to be the main source of differentiation in the efficiency of this technology. When sexed sperm is used the efficiency of IVF may be affected not only by bull effect but by differentiated susceptibility of bull spermatozoa to Hoechst 3342 staining as well (SMORĄG et al., 1999). The differentiated susceptibility of spermatozoa to the vital staining might be conditioned not only by specific bull effect but by differentiated sensitivity of sperm chromatin to denaturation as well (BOCHENEK et al., 2001; KĄTSKA-KSIĄŻKIEWICZ et al., 2005). In development of IVF procedure for sexed bovine sperm it seems interesting to determine the effect of sperm fraction on developmental competence of the in vitro produced embryos. It has been shown in IVF experiments that male embryos develop more rapidly and with higher efficiency in comparison to female embryos (AVERY et al., 1991; DOMINKO and FIRST, 1993; WAYDA et al., 1995).

The objective for these experiments was to compare the effectiveness of using fresh and/or frozen/thawed X- and Y-bearing fractions of flow-cytometrically sorted bovine spermatozoa for in vitro fertilization of bovine in vitro mature oocytes and subsequent blastocyst development.

Material and Methods

In vitro oocyte maturation. Bovine ovaries were collected at the local abattoir and were transported to the laboratory at 28 to 30 ºC within 2 to 3 h of slaughter. The ovaries were washed three times in warm PBS supplemented with 0.075 g kanamycin l^{-1}. Cumulus-oocyte complexes (COCs) were freed from ovaries following the isolation and subsequent rupture of vesicular follicles 2 to 8 mm in diameter in manipulation medium (KĄTSKA et al., 1996). The manipulation medium consists of TCM-199, Earle's salt with glutamine and without sodium bicarbonate, buffered with 25 mM Hepes and supplemented with 10% fetal calf serum (FCS). Oocytes with compact cumulus and evenly granulated cytoplasm were cultured in 2 ml of TCM-199 (Earle's salt, buffered with sodium bicarbonate, pH 7.4) supplemented with 20% estrus cow serum (ECS, heat inactivated) and an additional 3 to 5 x 10^6 granulosa cells ml^{-1}.

Oocytes were cultured for 22 to 23 h at 38.5 ºC at humidified atmosphere (KĄTSKA et al., 1996). To eliminate cow effect on in vitro-fertilization results, oocytes recovered from ovaries of slaughtered animals were pooled and used for in vitro maturation, and after maturation oocytes were randomly selected for IVF with sorted and unsorted sperm.

Sperm preparation and IVF. Sperm from 6 bulls (at least 3 replicates per bull) obtained from Polish animal breeding stations were used for the experiment. For each trial sorted and unsorted sperm samples of the same bull were simultaneously used for in vitro-fertilization of matured oocytes. All semen samples were characterized by similar morphological parameters. Sperm was sorted in a MoFloSX cytometer (Cytomation) using the method of the XY Inc., Colorado, USA (Research Collaboration Agreement). After sorting, the sperm was either used for IVF immediately or frozen (following the XY Inc. (Colorado, USA) protocol) and stored in liquid nitrogen up to the time it was used for IVF. Simultaneously with the sorted sperm, unsorted, fresh or frozen/thawed sperm was used for IVF as a control. Small aliquots of frozen/thawed fractions were used for flow cytometric sort reanalysis to determine the sort purities of the putative X and Y sperm populations. Reanalysis revealed a satisfactory purity of sorted fractions oscillating between 81 to 82 %.

The standard protocol of sperm capacitation (KĄTSKA-KSIĄŻKIEWICZ et al., 2005) was applied for both control, fresh and frozen/thawed sperm and for fresh fractions of sexed sperm. Briefly, -sperm was separated on a discontinuous Percoll (Pharmacia, Uppsala, Sweden) density gradient (1 ml 45% Percoll over 1 ml 90% Percoll) by centrifugation for 30 min at 300 x g at room temperature, washed in calcium ion-free TALP medium (PARRISH et al., 1988) and pelleted by centrifugation at 100 x g for 10 min. Spermatozoa were counted in a hemocytometer and diluted up to approx. 3-5 x 10^7 sperm/ml in the calcium ion-free TALP medium. This suspension was added to fertilization drops of Tyrode's albumin-lactate-pyruvate (TALP)-IVF containing 10 µg heparin ml^{-1} and mixture of penicillamine (20 µM; Sigma), hypotaurine (10 µM; Sigma), and epinephrine (1 µM; Sigma) at a concentration of 1 to 2 x 10^6 spermatozoa ml^{-1} of medium.

Frozen/thawed fractions of sorted spermatozoa were centrifuged after thawing in calcium ion-free TALP medium (500 g for 10 min) and immediately introduced into the IVF drops at 2 to 3 x 10^6 spermatozoa ml^{-1} of medium.

Mature COCs were washed and partially deprived of expanded cumulus cells before being transferred in groups up to 10 into 40 µl fertilization drops of TALP-IVF medium. Gametes were incubated together for 20 to 22 h at 39 ºC under 5% CO_2 in air.

Embryo culture and evaluation. After 22 to 24 h of gametes incubation, the oocytes were washed, freed of attached cells and transferred into 40 µl drops of B_2 medium (C.C.D., Paris, France) under mineral oil for 20 to 24 h (40 to 44 h post insemination). Then, uncleaved ova were discarded and embryos were placed in co-culture with Vero cells in 100 µl of B_2 medium supplemented with 2.5% fetal calf serum (Sigma) into 4-well dish for a further 6 to 8 days, i.e., up to the blastocyst or to the hatched blastocyst stage (KĄTSKA et al., 1998). Medium in co-culture was partially changed every 48 h.

Recovered blastocysts (mainly originated from IVF with X-bearing sperm) were frozen and stored in liquid nitrogen for further transfer application; the hatched

blastocysts (mainly originated from Y-bearing sperm) were used for sex identification by PCR technique using method described by WAYDA et al. (1995).

Statistics. Statistical analysis was performed with 3-factoral analysis of variance using GLM procedure of SAS software, for calculations all data were logarithmically transformed. Individual groups were compared with LS-means method.

Results

A total of 2712 IVM oocytes were fertilized with both fresh and frozen-thawed sexed and control sperm of 6 bulls (Table 1).

Table 1
Cleavage and embryonic development of IVM oocytes fertilized with sorted and unsorted sperm of different bulls (Furchungsrate und Embryonenentwicklung in vitro gereifter Oozyten nach Befruchtung mit geschlechtsdeterminierten und nichtdeterminierten Spermien verschiedener Bullen)

Bull	Sperm sample	No of oocytes for IVF	Cleaved embryos No (%)	Blastocysts No (%)
BENELUX	Control fresh	117	41 (35.0)	7 (17.1)
	X fresh	173	41 (23.7)	5 (12.2)
	Y fresh	117	21 (17.9)	5 (23.8)
	Control frozen/thawed	111	40 (36.0)	7 (17.5)
	X frozen/thawed	151	18 (11.9)	1 (5.6)
	Y frozen/thawed	168	12 (7.1)	0
ELEGANT	Control fresh	31	28 (90.3)	13 (46.4)
	X fresh	-	-	-
	Y fresh	32	9 (28.1)	5 (55.6)
	Control frozen/thawed	37	17 (45.9)	6 (35.3)
	X frozen/thawed	111	28 (25.2)	2 (7.1)
	Y frozen/thawed	143	49 (34.3)	9 (18.4)
BEST	Control frozen/thawed	105	76 (72.4)	10 (13.2)
	X frozen/thawed	115	58 (50.4)	11 (19.0)
	Y frozen/thawed	91	41 (45.0)	8 (19.5)
77	Control fresh	93	61 (65.6)	9 (14.8)
	X fresh	64	11 (17.2)	1 (9.1)
	Y fresh	56	18 (32.1)	2 (11.1)
RISIKO	Control fresh	70	34 (48.6)	9 (26.5)
	X fresh	50	16 (32.0)	1 (6.2)
	Y fresh	62	18 (29.0)	3 (16.7)
	Control frozen/thawed	82	23 (28.0)	2 (8.7)
	X frozen/thawed	121	33 (27.3)	4 (12.1)
	Y frozen/thawed	99	16 (16.2)	3 (18.8)
EPIGRAM	Control frozen/thawed	146	98 (67.1)	5 (5.1)
	X frozen/thawed	257	119 (46.3)	10 (8.4)
	Y frozen/thawed	143	51 (35.7)	8 (15.7)

The results of fertilization and embryonic development showed large variations among ejaculates of different bulls. Therefore, on the basis of the kind of sperm sample used for IVF, all results were summarized as presented in Table 2. There were differences in cleavage rates among fresh, control sperm (52.7%), X fraction (26.8%) and Y fraction (24.7%). Similar differences in cleavage rates were shown for frozen/thawed control sperm (52.8%), X fraction (33.9%) and Y fraction (26.2%) (Table 2).

Statistical 3-factoral analysis of variance with logarithmically transformed data showed no interaction between bull x sperm freezing, bull x sperm sexing and sperm

freezing x sperm sexing. Embryo cleavage rates were significantly affected by bull (P<0.0001), sperm sexing (P<0.0001) and sperm freezing (P<0.01). Blastocysts development was affected by sperm freezing (P<0.04) and sperm sexing (P<0.01). The significant differences were shown between unsorted and sorted sperm, however no differences in embryo cleavage rates and blastocysts rates were observed between X- and Y-sperm fractions, both fresh and frozen/ thawed (Table 3).

Table 2
Summarized data of cleavage and embryonic development of IVM oocytes fertilized with sorted and unsorted sperm (Zusammengefasste Daten der Furchungsraten und Embryonalentwicklung in vitro gereifter Oozyten nach Befruchtung mit geschlechtsdeterminierten und nichtdeterminierten Spermien)

Sperm Sample	No of oocytes for IVF	Cleaved embryos No (%)	Blastocysts No (%)
Control fresh	311	164 (52.7)	38 (23.2)
X fresh	254	68 (26.8)	7 (10.3)
Y fresh	267	66 (24.7)	15 (22.7)
Control frozen/thawed	481	254 (52.8)	30 (11.8)
X frozen/thawed	755	256 (33.9)	18 (13.1)
Y frozen/thawed	644	169 (26.2)	28 (16.6)

Table 3
The 3-factoral analysis of variance (F- Fisher's test; P-probability) (Die 3-Faktorielle Varianzanalyse (F - Test; P - Wert))

Factor	Significance of differences (F; P)	
	Cleavage rate	Blastocysts rate
Bull	7.40; 0.0001**	2.10; 0.0736
Sperm freezing	6.70; 0.0114*	4.05; 0.0475*
Sperm sexing	9.89; 0.0001**	4.55; 0.0134*

Comparisons of bull effect on embryo cleavage rates (Table 4) showed significant differences among bulls: Benelux and Best (P<0.0001); Benelux and Elegant (P<0.0005); Benelux and Epigram (P<0.0001); Best and Risiko (P<0.003); Best and 77 (P<0.02); Epigram and 77 (P<0.01); Epigram and Risiko (P<0.0009); Elegant and Risiko (P<0.04).

Table 4
Statistical differences between bulls in embryo cleavage rates (Statistische Unterschiede zwischen den Bullen bei der Teilungsrate der Embryonen)

Bull	LS-mean	77	Benelux	Best	Elegant	Epigram	Risiko
77	3.259966	-	0.2408	0.0254*	0.1095	0.0144*	0.9438
Benelux	2.932192	0.2408	-	0.0001**	0.0005**	0.0001**	0.0700
Best	4.063806	0.0254*	0.0001**	-	0.3831	0.8428	0.0033**
Elegant	3.816325	0.1095	0.0005**	0.3831	-	0.2681	0.0405*
Epigram	4.114783	0.0144*	0.0001**	0.8428	0.2681	-	0.0009**
Risiko	3.280925	0.9438	0.0700	0.0033**	0.0405*	0.0009**	-

Comparisons of bull effect on blastocyst rates (Table 5) showed significant differences among bulls: Benelux and Best (P<0.007); Benelux and Elegant (P<0.01) and Best and Risiko (P<0.03).

The analysis of sex ratio of the hatched blastocysts (n=21) derived from oocytes fertilized in vitro with Y fraction of sorted sperm showed 76.2% males (n=16) and 23.8% (n=5) females.

Table 5
Statistical differences between bulls in blastocyst rates (Statistische Unterschiede zwischen den Bullen bei der Blastozystenrate)

Bull	LS-mean	77	Benelux	Best	Elegant	Epigram	Risiko
77	1.405487	-	0.9105	0.0747	0.1119	0.3963	0.7822
Benelux	1.363157	0.9105	-	0.0078**	0.0179*	0.1614	0.5518
Best	2.267778	0.0747	0.0078**	-	0.7647	0.1805	0.0348*
Elegant	2.153120	0.1119	0.0179*	0.7647	-	0.3318	0.0709
Epigram	1.799629	0.3963	0.1614	0.1805	0.3318	-	0.3916
Risiko	1.516677	0.7822	0.5518	0.0348*	0.0709	0.3916	-

Discussion

An attractive feature of IVF is that fewer sexed sperm are needed than for artificial insemination. In our preliminary trials aiming to develop the procedure for capacitation of flow cytometrically sorted sperm (data not shown) it has been found that standard capacitation protocol used in our laboratory for frozen/thawed sperm (KĄTSKA-KSIĄŻKIEWICZ et al., 2005) might be successfully applied for fresh sperm, both sorted and unsorted. However, the sorted and then frozen/thawed fractions of sperm poorly survived after the capacitation treatment and, moreover, only few sperm could be recovered following Percoll's separation. These observations are in agreement with conclusions of LU et al. (1999) that sperm sexed by flow cytometry are probably precapacitated, necessitating modifications to standard IVF systems. For these reasons we decided to simplify the procedure of capacitation for frozen/thawed fractions of sorted sperm. However, the applied modification limiting preparation of these fractions only to a centrifugation of thawed sperm did not allow removing dead spermatozoa that had been damaged following the cryoconservation procedure, which might exert a detrimental effect on fertilization results.

Several authors dealing with in vitro embryo production in cattle (MILLER and HUNTER, 1987; AOYAGI et al., 1988; EYESTONE and FIRST, 1989; GALLI and LAZZARI, 1996; KĄTSKA et al., 1996; CHAMBERLAND et al., 2001; GALLI et al., 2003; KĄTSKA-KSIĄŻKIEWICZ et al., 2005) have described the variability among bulls in the ability of their sperm to become capacitated, fertilize oocytes and subsequently produce competent embryos developing to the blastocyst stage. In terms of factors affecting the outcome of IVF, bull variability has been the main source of difficulty in the work. These bull-to-bull variations in the outcome of in vitro embryo production frequently lead to selection of bulls used for IVF. However, even in the case of a severe bull selection, the developmental capacity of in vitro produced embryos is still lower in comparison to their in vivo counterparts (LEIBFRIED-RUTLEDGE et al., 1987; LONERGAN et al., 2003; GALLI et al., 2003) and may be numerically further reduced when sorted sperm is used for fertilization (CRAN et al., 1993; 1994; LU et al., 1999; ZHANG et al., 2003; 2005). However, as we demonstrated in the present experiment, the reduction of developmental competence of embryos obtained with sorted sperm concerned mainly cleavage rates and less affected the blastocyst rates. Indeed, similar rates of blastocyst per cleaved embryo were produced using the control sperm and Y chromosome-bearing sperm, both fresh and frozen/thawed. In contrast, using fresh fractions of X chromosome-bearing sperm significantly less blastocysts per cleaved embryos were obtained in comparison to the control and to the Y chromosome-bearing sperm.

Several studies have demonstrated that male bovine embryos produced in vitro develop faster than female embryos produced in vitro, which results in more male than

female blastocysts (DOMINKO and FIRST, 1993; GALLI et al., 2003). Comparing the rate of blastocyst development of male and female bovine embryos derived from sexed sperm and cultured in vitro to blastocyst stage we noticed that the differences occurred only in the case of fresh, sorted sperm. This fact needs further investigations to determine the reasons for both reduced cleavage rates of embryos obtained with sorted fractions and the lower developmental competence of female embryos produced with fresh X fractions of sorted sperm. Similar lower cleavage rates for sperm sorted with the same procedure (XY Inc.) in comparison to control sperm and lack of significant differences in blastocyst rates were observed by ZHANG et al. (2003).

There is now a growing amount of evidence to suggest that while bull variability can impact on developmental competence of embryos, the intrinsic quality of the oocyte is the key factor determining the proportion of oocytes developing to the blastocyst stage (LONERGAN et al., 2003). This fact should also be taken into account when considering the total efficiency of the applied procedures. To avoid cow effect of in vitro embryo production, oocytes, recovered from randomly collected ovaries of slaughtered animals, were pooled for IVM and, following maturation, again subdivided for IVF with different sperm samples.

In trying to establish accuracy of sperm sorting we carried out the sex determination of the hatched male blastocysts using PCR technique. The PCR analysis of sex of hatched male blastocysts revealed 76.2% accuracy. We speculated that this ratio was due to the small number of embryos analysed by PCR and, with a larger number of blastocysts the rate of pre-sexing accuracy might be more precisely and objectively determined. However, in the future experiments, this ratio has to be improved to gain more benefits from sorting on the number of produced offspring of a certain sex. On the other hand, in the field experiments when X chromosome-bearing fraction of sorted sperm of several bulls - including the bulls used for the in vitro fertilization trials in this experiment – were used for insemination 86.5% accuracy (45 females out of 52 calves born) has been observed, which seems to be quite satisfactory (SMORĄG et al., 2005).

Our results suggest that there were significant differences in cleavage rates due to sperm sorting in comparison to control, unsorted sperm and no differences between effectiveness of X and Y fractions of spermatozoa. The fertilization and embryo development rates with sorted and unsorted sperm were variable among bulls, which indicates that sorting high quality sperm from specific bulls might increase the efficiency of embryo production of a desired sex.

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