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Original Scientific Article

IMPROVEMENT OF SPERM SORTING EFFICIENCY AND FERTILIZING CAPACITY EMPLOYING TWO VARIATIONS OF A NEW BULL SEMEN EXTENDER (SEXCESS®)

Primož Klinc[1]*, Detlef Rath[2]

[1]Clinic for Reproduction and Horses, Veterinary Faculty, Gerbičeva 60, 1000 Ljubljana, Slovenia; [2]Friedrich-Loeffler-Institut, Institute of Farm Animal Genetics Hoeltystr. 10, 31535 Neustadt, Germany

*Corresponding author, E-mail: primoz.klinc@vf.uni-lj.si

Summary: The aim of the present study was to analyse the effect of temporary chemical inhibition of sperm motility before and during flow cytometrical sorting on the quality and fertilizing capacity of sex sorted and frozen/thawed spermatozoa. The quality and fertilizing capacity of temporarily immobilized spermatozoa sorted in the presence of sodium fluoride (S-AO-NF) was compared with sorted semen using a standard protocol as developed in our laboratory (S-AO) and with unsorted control (C). Semen from two bulls (Holstein Friesian and Limousine) was used and in total 283 first inseminations with 2 million live, frozen/thawed sex sorted and unsorted spermatozoa were performed on 197 farms. Motility of spermatozoa was completely inhibited after addition of sodium fluoride and after sorting and centrifugation returned to a similar degree as before. Motility after thawing and incubation at 37 °C did not differ between groups. After further incubation at 37 °C for 12 h and 24 h motility was significantly higher ($P<0.001$) in both sorted groups compared to unsorted semen. The percentage of spermatozoa with acrosome abnormalities in frozen/thawed samples was significantly higher ($P<0.05$) in S-AO and C group when compared to S-AO-NF group. The same difference was found for morphological sperm abnormalities. The percentage of acrosome reacted and viable spermatozoa was analysed by FITC-PNA/PI staining. The percentage of acrosome reacted spermatozoa was significantly lower ($P<0.001$) in both groups of sorted semen when compared to unsorted control. The percentage of viable spermatozoa was significantly higher ($P<0.05$) in the S-AO-NF group when compared to groups S-AO and C. Pregnancy rates after artificial insemination with the frozen/thawed semen were not significantly different between sorted spermatozoa and controls (72.7 % vs. 73.3 % vs. 79.2 % for S-AO-NF, S-AO and C respectively).

Key words: semen; spermatozoa; flow cytometry; sorting; sex; freezing / thawing; artificial insemination; pregnancy

Introduction

Like in other domestic animal species, a broader utilisation of sexed sperm would be very beneficial for cattle industry. So far, only flow cytometry allows producing a significant shift of sex ratios. However, the technique still has its limitations in sorted sperm output (1, 2), although many improvements have helped in the recent years to start first commercial applications as shown in UK and Texas, USA.

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Especially introduction of high speed flow cytometry (3), better orientation of spermatozoa in front of the Laser beam (4) and improvements in sperm preparation before and after sorting by special media components (unpublished data) allow to minimize the sperm concentration and use liquid as well as frozen/thawed spermatozoa. However, fertilizing potential of sorted frozen/thawed spermatozoa has not reached the quality of unsorted analogues (5). The lifetime after sorting and freezing/thawing is limited and even with adapted insemination protocols, the number of calves born per AI or number of AI necessary to get the same fertility rates are critical (5). A potential solution might be to

safe as much energy as possible during processing and sorting. This might be achieved with reversible, chemical inhibition of the sperm metabolism.

Ornidazone (6), bromoydroxypropanone (7), nitric oxide (8), alfa-chlorhydrin (9), gossypol (10), Cytochalasin B (11), formaldehyde (12) and sodium fluoride (13, 14) are examples for chemicals, known to have an inhibiting effect on sperm motility. Dott et al. (12) were able to achieve pregnancies after artificial insemination in ewes and sows with formaldehyde-immobilized spermatozoa. As the toxicity of formaldehyde is high (15), specific inhibition of sperm motility may be performed by sodium fluoride (13). Sodium fluoride is a conventional inhibitor of protein-thyrosine-phosphatase (16), which is included in the process of sperm capacitation and motility (17, 18) and was reported as immobilizing substance for bull spermatozoa (19).

Flow cytometrical sorting could benefit from temporarily inhibition of sperm activity not only for the prolongation of sperm lifetime, but also to get a better orientation of spermatozoa in front of the Laser beam and to reduce individual bull effects during staining and sorting. In the present study, we analysed the effect of temporary sperm immobilization with sodium fluoride on sorting efficiency, quality and fertility of frozen thawed bull spermatozoa.

Materials and methods

L-α-Lysophosphatidylcholine (L 5004); Lectin, FITC labelled from Arachis hypogaea (peanut) (L7381); Propidium iodide (P4170), 6-Carboxyfluorescein diacetate (C5041); Bisbenzemide H 33342 (B2261), Pyruvic Acid (P-3662) and Catalase (C-1345) were purchased from Sigma-Aldrich (Taufkirchen, Germany) all the other chemicals, if not specially stated, were purchased from Carl Roth GmbH + Co (Karlsruhe, Germany).

Methods

Semen ejaculates were collected from fertility proven and performance tested Holstein Friesian bull (Bull 1, 11 ejaculates) with high fertility rate and a young Limousine bull (Bull 2, 4 ejaculates) in performance test. Ejaculates that were included into research had to reach minimum criteria of at least 70% motility and 80% of morphological normal spermatozoa.

Ejaculates were kept in a water bath at 27°C and motility was estimated under phase contrast microscope (Olympus BX 60) at 37°C. Morphology was analysed after fixation in Hancock fixative (2.784 g Tri-Natriumcitrat-Dihydrat, 4 mL 37 % formaldehyde solution and bi-distillated water to 100 mL), within 4 hours after semen collection. Concentrations of spermatozoa in ejaculates were determined with a haemocytometer (Coulter counter®). Raw semen was divided into two parts and diluted to $1x10^8$ spermatozoa/mL either with the commercially available extender Sexcess® AX (Masterrind Verden, Germany), supplemented with antioxidants and BSA fraction V (group S-AO), or Sexcess® FX additionally containing sodium fluoride (group S-AO-FX). Spermatozoa were labelled with 15, 20 and 25 µL of a 8.12 mM Hoechst 33342 solution and incubated for 1 hour at 34°C. Thereafter, labelled semen samples were kept at 22°C in the dark and sorted within 7 hours after onset of incubation. The labelled semen samples were pre-tested for maximal separation with a flow cytometer and the concentration of Hoechst 33342 stain giving the best resolution of two sperm populations was used for daily sorting. The other part of the ejaculate, which was not used for sorting, was frozen following a routine protocol of a commercial AI center and served as control. Briefly, semen was gradually diluted with TRIS egg-yolk freezing extender I to the concentration of $26.4x10^6$ spermatozoa/ml, cooled to 5°C within two hours and further diluted with TRIS egg-yolk freezing extender II containing 0.75 % detergent (Equex STM®, Nova Chemical sales, Inc., Scituate, USA) and 12.8 % (v/v) of 87 % Glycerol to a concentration of $13.2x10^6$ spermatozoa / mL or $3.3x10^6$ spermatozoa / straw, filled into 0.25 mL straws (Minitüb, Tiefenbach, Germany), and frozen in liquid nitrogen (LN_2). Briefly, straws were placed on metal holder in nitrogen vapour 3-5 cm above LN_2 for 15 minutes. Frozen samples were then plunged into LN_2 and kept in the semen container (in LN_2) until used for analysis or artificial insemination.

Processing of the sorted spermatozoa

Sperm sorting was performed according to the Beltsville Sperm Sorting Technology (3). Sorted spermatozoa of group S-AO were collected into collection fluid (max. 8 million spermatozoa per tube) and then centrifuged at 838xg for 20 minutes. Centrifugation of sorted spermatozoa in group S-

AO-NF allowed lower force (500xg for 15 minutes) because cells were immobilized and gave a more distinct pellet. Supernatant was discharged and the remaining sperm pellet of both groups was diluted with extender Sexcess I® (Masterrind Verden, Germany) to a concentration of $41x10^6$ spermatozoa /mL and cooled to 5°C within 2 hours. Once 5°C was reached, semen samples were further diluted with extender Sexcess II® (Masterrind Verden, Germany) to a concentration of $20.5x10^6$ spermatozoa/mL. Plastic straws (0.25 mL) (Minitüb, Tiefenbach, Germany) were pre-filled with a first segment (50 µL of a mixture made from extender I and II), and with a second segment, filled with 160 µL sorted semen (3.3 millions spermatozoa in total, equivalent to approx. $2 x10^6$ life spermatozoa). Semen samples were frozen in liquid nitrogen as already described for control samples.

Sperm analysis of frozen thawed samples

Motility analysis

Sperm motility of raw semen, of sperm samples before and after sorting and after thawing was analysed. Prior to analyses samples were prewarmed to 37°C for 15 minutes and analysed under a phase-contrast microscope (OlympusBX60) at 100x magnification, equipped with heating plate to maintain 37°C. From each sample two 6 µL drops were transferred onto pre-wormed objective glass and covered with coverslip glass. At least three fields were evaluated per drop.

Morphology analysis

Morphology was analysed after fixation of the samples in Hancock solution under a phase-contrast microscope (Olympus BX 60) at 1000x magnification. At least 200 spermatozoa were examined per sample for morphological abnormalities (MAS) and acrosome integrity. Spermatozoa were divided into two groups. Spermatozoa that had any pathological change of acrosome were included into group named damaged acrosomes (DA). Group MAS corresponds to the percentage of spermatozoa with damaged acrosomes plus spermatozoa that had any other morphological abnormalities.

Acrosome integrity and membrane stability of spermatozoa (FITC-PNA/PI)

Acrosome integrity and membrane stability were analysed with FITC-PNA/PI as described previously (20). Pre-warmed Eppendorf cups were filled with 50 µL of semen and 1 µL FITC-PNA (2 mg FITC-PNA in 2 mL PBS) as well as 2 µL PI (1 mg propidium iodide in 10 mL physiological NaCl solution) were added. Samples were incubated at 38°C for 5 minutes and supplemented with 5 µL paraformaldehyde (1 % in PBS) immediately before microscopic examination. At least 200 spermatozoa were examined under a fluorescence and phase contrast microscope (Olympus BX 60; U-MNIB filter) at 400x magnification. Spermatozoa were divided into four groups: 1. PNA-negative/PI-negative (viable spermatozoa with intact acrosome); 2. PNA-negative/PI-positive (spermatozoa with damaged plasma membrane and intact acrosome); 3. PNA-positive/PI-positive (spermatozoa with damaged plasma membrane and reacted acrosome); 4. PNA-positive/PI-negative (spermatozoa with intact plasma membrane and reacted acrosome). Mean percentages of viable spermatozoa with intact membranes (group 1) and acrosome reacted spermatozoa (group 3 and 4) are presented in the results.

Artificial insemination and pregnancy control

All the straws were coded in order to make it impossible for inseminators to know the content. Straws were randomly distributed to three well experienced AI technicians within 1.5 month after sorting. For AI, semen samples were thawed at 37°C for 20 sec. and inseminated 12-24 hours after onset of natural oestrus. Technicians were advised to insert a normal AI catheter under rectal control as deep as possible into the uterine horn but without extra force or strong manipulation. In cases where it was difficult to determine the location and size of the follicle, semen was either deposited into the uterine body or the content of the straw was split for AI in both uterine horns. Pregnancies were controlled 30-60 days after insemination by transrectal examination and transrectal ultrasonography (Aloka®; 5 MHz).

Statistical analyses

Statistical analyses were performed with SIGMA STAT 2.03 for windows (Jandel Scientific Cooperation, San Ragael, CA, USA). Pregnancy rates were analysed with Chi-square test. Semen quality parameters were tested for normal distribution and either analysed with One-way ANOVA or ANOVA and Ranks and Tukey test. Data are expressed as percentages or means ± SD. Differences were considered to be significant at $P<0.05$.

Results

In the raw semen samples 72.1 ± 3.7 % spermatozoa were motile. After labelling and before sorting in the S-AO group 66.9 ± 4.6 % were motile, whereas all spermatozoa in the S-AO-NF group were immobilized. After sorting the percentage of motile spermatozoa in S-AO-NF group was 72.9 ± 4.9 %, whereas sperm motility in S-AO samples was 70.6 ± 5.6%. The difference was not significant ($P<0.05$). Sperm motility after thawing and incubation at 37°C for 6 h was not significantly different between groups. Further incubation of frozen thawed spermatozoa for 12 and 24 hours showed significant higher motility of spermatozoa in both sorted groups compared to unsorted standard processed semen (Table 1).

Table 1: Sperm motility of sorted and control groups immediately after thawing (0 h) and after further incubation at 37°C for 6, 12 and 24 h

	0 h (%)	6 h (%)	12 h (%)	24 h (%)
Control	61.5 ± 3.8	45.8 ± 9.3	$7.5 \pm 11.6^{a(L)}$	0.0 ± 0.0^{a}
S-AO	59.4 ± 4.2	45.6 ± 9.4	33.1 ± 6.5^{b}	$21.3 \pm 10.3^{b(L)}$
S-AO-NF	59.0 ± 5.9	47.9 ± 7.0	37.1 ± 4.9^{b}	$22.9 \pm 7.6^{b(L)}$

Values with different superscripts differ significantly ($P<0.001$).
Data are shown as mean ± SD. (L) - only local motile spermatozoa were observed.

After thawing the percentage of spermatozoa with damaged acrosomes and morphologically abnormal spermatozoa were significant lower in S-AO-NF group compared to S-AO group and unsorted control samples (Table 2).

Table 2: Percentage of spermatozoa with damaged acrosomes (DA) and morphologically abnormal spermatozoa (MAS)

	DA (%)	MAS (%)
Control	22.3 ± 4.0^{a}	29.0 ± 5.8^{a}
S-AO	23.0 ± 5.2^{a}	30.0 ± 6.7^{a}
S-AO-NF	14.7 ± 7.5^{b}	19.0 ± 8.1^{b}

Values with different superscripts differ significantly ($P<0.05$).
Data are shown as mean ± SD.

The percentage of acrosome reacted spermatozoa as seen after FITC-PNA staining was significantly lower in both sorted groups as compared to unsorted control samples. The percentage of viable spermatozoa was significantly higher in S-AO-NF group compared to S-AO group and unsorted control (Table 3).

Table 3: Percentage of acrosome reacted (AR) and spermatozoa with intact membranes (viable) in frozen/thawed samples, according to FITC-PNA/PI staining

	AR (%)	Viable (%)
Control	19.1 ± 4.7^{a}	61.4 ± 8.2^{c}
S-AO	10.6 ± 3.5^{b}	56.9 ± 4.7^{c}
S-AO-NF	6.7 ± 2.6^{b}	72.1 ± 5.0^{d}

Values with different superscripts are significantly different: a:b $P<0.001$; c:d $P<0.05$-
Data are shown as mean ± SD.

In total 283 inseminations were performed under field conditions on 197 Slovenian farms. Mostly it was one insemination per farm, than two inseminations were performed on 30 farms, 3 on 11 farms, 4 on 3 different farms and 5 inseminations on 1 farm. Table 4 shows the pregnancy results. No differences were found between sorted and unsorted semen samples or among sorted samples. Reanalysis of semen after thawing revealed 92.5 % average purity for X-chromosome bearing spermatozoa.

Table 4: Pregnancy rates after artificial insemination with sorted frozen/thawed spermatozoa diluted in Sexcess® AX or Sexcess® FX

	Control	Sexcess® AX (S-AO)	Sexcess® FX (S-AO-NF)
Bull 1(%)	75.5 %	73.5 %	62.1 %
Pregnant/all (n/n)	37/49	36/49	36/58
Bull 2 (%)	79.2 %	73.3 %	72.7 %
Pregnant/all (n/n)	42/53	32/44	22/30
Total (%)	77.5 %	73.1 %	65.9 %
Total (n/n)	79/102	68/93	58/88

No differences in pregnancy rates were seen among AI technicians (69.1, 70.1 and 77.9 %). Place of the semen deposition was recorded in 260 cases. It had a significant effect ($P<0.05$) on the pregnancy rates and were significantly lower after semen deposition into the uterine body and into uterine horn ipsilateral to the ovulation site compared to those inseminations where the content of the straw was divided and inseminated into both horns.

Table 5: Effect of place of semen deposition on pregnancy rates

AI place	all/pregnant	%
Uterine body	50/33	66.0[a]
Ipsilateral to ovulation	130/88	67.7[a]
both horns	80/66	82.5[b]

Values with different superscripts are significantly different: a:b $P<0.05$

Discussion

In the present study, we have demonstrated that optimal handling according to the modified Beltsville Sperm Sorting Technology (3) and protection during staining, sorting, centrifugation, diluting and freezing of the sorted spermatozoa leads to high quality of sexed semen and to pregnancy rates that are comparable with those of unsorted controls after artificial insemination under normal field conditions. However, it has to be mentioned that all experiments were performed with semen from selected bulls. Selection criteria were that non return rates were above average and that staining and sorting was homogenous between collection days in order to speed up the sorting process.

Previous studies demonstrated lower pregnancies rates after artificial inseminations with flow cytometrically sorted spermatozoa when compared to unsorted control. Probable reasons were induction of capacitation, membrane damages (21, 22, 23) and rapid loss of motility (24). In consequence, insemination protocols had to be changed and insemination had to be performed closer to ovulation.

In the present study, lifetime of sorted spermatozoa was at least as long as for those in the control group. The percentage of motile spermatozoa was even higher in both groups of sorted semen compared to unsorted controls after incubation at 37°C for 12 and 24 h. This was probably caused by utilization of antioxidants, preventing generation of reactive oxygen species, oxidation of membrane lipids, and loss of motility due to oxidation (25). Addition of sodium fluoride caused a complete, reversible inhibition of sperm motility and seemed to reduce interference of mitochondria in the mid piece of the sperm tail with the high voltage electric field, necessary to separate both sperm populations. Sorted spermatozoa regained their motility after sorting and centrifugation completely, which shows the complete reversibility of motility inhibition with sodium fluoride.

Chinoy and Naraya (26) reported toxic effects of sodium fluoride on human spermatozoa, causing irreversible damages like elongated heads, deflagellation, acrosome loss and coiling of the tails. However, in the present study, concentration of sodium

fluoride was much lower and enabled spermatozoa to completely regain motility. So far, no negative effects of sodium fluoride were seen. In opposite, the highest sperm membrane integrity was found in those samples that were diluted with Sexcess® FX. In addition, these samples also showed better sperm quality after sorting, which might mainly be due to shorter centrifugation time and force.

Laboratory results were confirmed by pregnancy data that were obtained from an AI field trial in heifers. No significant differences were seen between inseminations with sorted vs. unsorted semen.

The handling was as easy as with unsorted semen and no significant effect on the insemination results were found among AI technician. However, the site of semen deposition in the female genital tract had significant effect on pregnancy rates. Williams et al. (27) reported reduced fertility after cervical deposition of the semen compared to uterine body or bicornual insemination, and Senger et al. (28) saw significantly more pregnant animals after bicornual insemination compared to insemination into the uterine body. Accordingly, in the present study significantly more animals became pregnant after bicornual compared to uterine body insemination. This is probably because more spermatozoa were lost during transport to the fertilization site after they were deposited into the uterine body. It is also interesting that lower pregnancy rates were obtained after semen was deposited into the ipsilateral uterine horn compared to the bicornual insemination. We believe that the reason could be due to the failure of correct diagnosis of ovulation site and consequently more inseminations were performed into the wrong uterine horn. It is also possible that the ovary (follicle) was damaged when the inseminator diagnosed the site of follicle growth.

In conclusion, the use of Sexcess® extender to optimize bull sperm quality has successfully been approved and improved the fertilizing ability of sexed spermatozoa to a comparable status of unsorted frozen/thawed bull semen. Further studies will have to analyse more intensively bull effects to broaden the number of males to be included in a sperm sorting based breeding programme.

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IZBOLJŠANJE USPEŠNOSTI LOČEVANJA IN OPLODITVENE SPOSOBNOSTI PO SPOLU LOČENIH SEMENČIC Z UPORABO DVEH OBLIK NOVEGA RAZREDČEVALCA ZA BIKOVO SEME (SEXCESS®)

P. Klinc, D. Rath

Povzetek: V raziskavi smo ugotavljali vpliv začasne kemične zaustavitve gibljivosti semenčic na kakovost in oploditveno sposobnost sortiranih in zamrznjenih/odmrznjenih semenčic. Vpliv smo ugotavljali pred in med postopkom ločevanja semenčic po spolu s pomočjo pretočne citometrije. Kakovost in oploditveno sposobnost semenčic, ki so bile začasno imobilizirane s pomočjo natrijevega fluorida (S-AO-NF), smo primerjali z rezultati, pridobljenimi pri semenu, ki smo ga sortirali po standardnem postopku, razvitem v našem laboratoriju (S-AO), ter kontrolnim nesortiranim semenom (C). V poizkus smo vključili seme dveh bikov. Na 197 kmetijah smo opravili 283 prvih osemenitev z 2 milijonoma živih odmrznjenih sortiranih in nesortiranih semenčic. Gibljivost semenčic, ki se je popolnoma zaustavila po dodatku natrijevega fluorida, se je po sortiranju in centrifugiranju vrnila na predhodno stopnjo. Po tajanju in inkubaciji na 37 °C med skupinami ni bilo statistično značilne razlike v gibljivosti semenčic. Po nadaljnji 12- in 24-urni inkubaciji na 37 °C pa je bila gibljivost semenčic statistično značilno višja ($P<0.001$) pri sortiranem semenu v primerjavi s kontrolnim semenom. Pri odmrznjenem semenu je bil odstotek semenčic s poškodovanimi akrosomi statistično značilno višji ($P<0.05$) v skupinah S-AO in C v primerjavi s skupino S-AO-NF. Pri ugotavljanju skupnih morfoloških sprememb smo prav tako ugotovili statistično značilno ($P<0.05$) višji odstotek spremenjenih semenčic v skupinah S-AO in C v primerjavi s skupino S-AO-NF. S pomočjo barvanja FITC-PNA/PI smo ugotavljali odstotek semenčic z reagiranimi akrosomi in odstotek vitalnih semenčic. V obeh skupinah sortiranega semena smo ugotovili statistično značilno nižji ($P<0.001$) odstotek semenčic z reagiranimi akrosomi v primerjavi z ne sortiranim semenom. Odstotek vitalnih semenčic je bil statistično značilno višji ($P<0.05$) v skupini S-AO-NF v primerjavi s skupinama S-AO in C. Po umetni osemenitvi z zamrznjenim/odmrznjenim semenom nismo ugotovili statistično značilne razlike v oploditveni sposobnosti med posameznimi skupinami sortiranega semena in kontrolnim semenom (72.7 % proti 73.3 % proti 79.2 % za skupine S-AO-NF, S-AO in C). Kljub temu da je bil spol potomcev statistično značilno ($P<0.05$) spremenjen v obeh skupinah sortiranega semena, je bila sprememba statistično značilno ($P=0.04$) višja pri skupini S-AO-NF v primerjavi s skupino S-AO.

Ključne besede: seme; semenčice; pretočna citometrija; ločevanje; spol; zamrzovanje / tajanje; umetno osemenjevanje; brejost