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Original Research Paper

EFFECTS OF PROCESSING PROCEDURES AFTER FLOW SORTING TO SEX BOVINE SPERMATOZOA AND CRYOPRESERVATION ON SPERM QUALITY AND FERTILITY

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Summary: The objectives of the study were to analyse the effects of semen processing after flow-cytometrical sorting and subsequent freezing in liquid nitrogen on the quality and fertilizing capacity of frozen/thawed spermatozoa. Quality of the frozen/thawed semen was evaluated by motility estimation, morphology analysis, membrane stability (6-CFDA/PI) test, capacitation test (FITC-PNA/PI staining with addition of L-α-Lysophosphatidylcholine) and fertility assessment in an insemination trial on a farm. Prolonged high dilution of flowcytometrically sorted spermatozoa before freezing had a significant negative effect on motility (P<0.001) and capacitation status (P<0.05). Positive effects on membrane stability were seen when glycerol was added at 5 °C shortly before straws were frozen compared to a system where glycerol was added before the equilibration process at room temperature (P<0.001). Independently of sperm processing after sorting, sexed spermatozoa had significantly more damaged acrosomes and morphological abnormalities (P<0.001). In addition sex sorted frozen/thawed spermatozoa (immediate centrifugation and glycerol addition at 5 °C) and unsorted frozen/thawed semen samples were submitted to a thermo-resistance test at 37 °C. Immediately after thawing no significant difference was seen in the percentage of motile spermatozoa between sorted and unsorted semen samples. However, after further incubation at 37 °C, motility of sorted spermatozoa was significantly lower than unsorted spermatozoa after 3 h (P<0.001) and 6 h (P<0.05). The pregnancy rates after insemination with sex-sorted and unsorted spermatozoa from the same ejaculates was tested in a field trial in heifers and cows with natural and synchronised oestrus cycles. In natural oestrus, more animals became pregnant after artificial insemination with unsorted than with sex-sorted spermatozoa (56.5 % vs. 17.6 %; P<0.001) No significant differences were observed between unsorted and sex-sorted frozen/thawed semen samples after artificial insemination of the animals with synchronised oestrus (36.4 % vs. 21.3 %; P=0.076). There was a significant effect of the bull on the pregnancy rates after artificial insemination with sex-sorted spermatozoa (P<0.05).

Key words: semen - analysis; spermatozoa - physiology - ultrastructure; quality control; semen preservation; insemination, artificial - veterinary - methods; pregnancy, animal; pregnancy rate; cattle - male

Introduction

Flowcytometrically sorted frozen/thawed spermatozoa have been successfully applied to artificial insemination in cattle (1). However, semen quality and pregnancy rates after artificial insemination were rather low compared to unsorted frozen/thawed semen (2-4). Main sources that may reduce the semen quality during flowcytomerical sorting are:

1. UV-Laser (5;6),
2. Pressure (7),
3. Dilution effects (8) and
4. Electric charge/electro-static field.

The dye itself at concentrations used for sorting does not have a significant impact on fertility of boar semen (9). Exposure to laser light accelerated Ca^{2+} transport into irradiated bull spermatozoa (10), enhanced Ca^{2+} binding to plasma membranes and

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inhibited Ca^{2+} uptake by mitochondria (5). Because of these insults the processing of sorted spermatozoa before freezing needs to be adjusted in order to obtain high quality and fertility rates after insemination.

Additionally, spermatozoa are highly diluted during sorting ($8x10^5$ spermatozoa/1 mL) by the sheath fluid to approximately one million spermatozoa/mL in the collection tube. High dilution was found to be detrimental for sperm motility and viability and could also affect the fertilizing capacity of spermatozoa (11, 12). For further processing, sexed sperm samples have to be centrifuged. Centrifugation however, increases the production of reactive oxygen species, which are detrimental for the sperm viability (13). As sorted spermatozoa are sensitive to membrane insults that may occur during centrifugation and storage at room temperatures, one objective of the present study was to test whether it is better to keep the highly diluted sorted spermatozoa at room temperature for a few hours and centrifuge all sorted samples together after 4 to 6 h or to perform centrifugation immediately after sorting, chilling and freeze each sample separately.

Centrifuged spermatozoa need to be diluted in a suitable extender, cooled to 5 °C and frozen in liquid nitrogen. The semen extender has to provide a suitable environment for survival and maintaining the viability of spermatozoa. The composition of semen extenders is mainly based on an energy resource (sugars such as glucose, fructose, lactose) and a buffer medium of different inorganic or organic salts. Additionally, the semen extenders contain components such as egg yolk, skim milk, specific amino acid, glycerol, detergent and antioxidants, which protect the spermatozoa (14). Egg yolk is an important component of semen extenders. The protective action of egg yolk is assumed to be due to membrane protection by low density lipoproteines (15), and the antioxidant ability of the phosphoprotein Phosvitin, which serves as an iron-carrier and protects spermatozoa against the Fenton reaction (16). Recently, egg-yolk of freezing extender for bull semen has been successfully replaced with soybean extract (17), but Tris egg-yolk freezing extenders are still preferred for freezing sorted bull semen (18). Glycerol is a cryoprotector, which is toxic to spermatozoa. Its toxicity is temperature dependent as seen especially in human semen (19). Some authors suggest the addition of glycerol after cooling the semen to 5 °C (14), whereas others prefer room temperature (20, 21). Therefore the second goal of our research was to determine the effect of glycerol addition at different temperatures.

Material and methods

Materials

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

Composition of solutions

Tris-sample fluid: 200.0 mM Tris-hydroxymethyl-aminomethan, 64.7 mM citric acid monohydrate, 95.5mM D-fructose and 50mg Gentamicin sulfate diluted in double distilled water.

Hoechst 33342 working solution: 8.9 mM of 2-(-4-ethoxyphenyl)-5-(4-methyl-1-piperazinyl)-2.5-bi-1H-benzimidazole in double distilled water.

Tris-sheath fluid: 197.1 mM Tris-hydroxymethyl-aminomethane, 55.3 mM citric acid monohydrate, 47.5 mM D-fructose, 0.058 mg Penicillin G and 0.050 mg Streptomycin sulfate diluted in 5 L double distilled water.

Hancock solution: 2.784 g Tri-sodiumcitrate-dihydrate, 4 mL 37 % formaldehyde solution and double distilled water to 100 mL.

Tris egg-yolk freezing extender I: mixture of 67.2 mL stock solution (297.6 mM Tris-hydroxymethyl-aminomethan, 96.3 mM Citric acid monohydrate, 82.6 mM D-fructose, 0.606 g Penicillin and 1.480 g Streptomycin sulfate per 1 L double distilled water.), 20.0 mL egg-yolk and 12.8 mL double distilled water.

Tris egg-yolk freezing extender II: the same as extender I, except that 12.8 mL double distilled water was replaced with 12.8 mL of 87 % glycerol.

Processing of semen

Semen was collected twice per week from two fertility proven Holstein Friesian bulls with an artificial vagina and kept in a water bath at 27 °C. Motility was estimated under a phase contrast microscope at 37 °C, and only ejaculates with >70 % total motility were used in the experiment. Concentration of ejaculated spermatozoa was determined with a

Thoma counting Chamber (Thoma neu®, Hecht, sontheim, Germany). One part of the raw semen was diluted with Tris-sample fluid to $1x10^8$ spermatozoa per millilitre and divided into three subsamples. Diluted samples were labelled with 15, 20 and 25 µL of 8.12 mM Hoechst 33342 solution and incubated at 37 °C for 1.5 h. The labelled samples were pre-tested with a flowcytometer, and the concentration of Hoechst 33342 stain giving the best resolution of the two sperm populations was used for daily sort. Labelled and incubated semen samples were kept at 22 °C in the dark and sorted within 7 h after onset of incubation. The remaining unlabelled part of the ejaculates was frozen according to a standard protocol described by Thun et al. (17). All semen samples were gradually diluted (by dripping) the same amount of TRIS egg-yolk freezing extender I and II, giving 6.4% final concentration of glycerol. Extender II was added to the semen, depending on the experimental design, at room temperature or at 5 °C. Final sperm concentration was set to $13.2x10^6$ spermatozoa/ml or $3.3x10^6$ spermatozoa/straw. Diluted semen was then filled into 0.25 mL straws (Minitüb, Tiefenbach, Germany), cooled to 5 °C within 2 h and frozen in liquid nitrogen. Freezing of the samples was performed in closed Styrofoam box (30 cm x 40 cm x 85 cm = high x bright x length). Briefly, straws were placed on metal holder in nitrogen vapour 3-5 cm above LN2 for 15 min. Frozen samples were than plunged into LN_2 and kept in the semen container (in LN_2) until used for analysis or artificial insemination.

Sperm Sorting

Sperm sorting was performed according to the Beltsville Sperm Sorting Technology (22). Semen samples, prepared as described above, were filtered through a 51 µm Cell Strainer grid (Falcon Becton Dickinson and Company, Franklin Lakes, NY, USA) and then supplemented with 1 µL food dye solution FD&C#40 (Warner Jekinson Company Inc. St. Louis, MO USA). Sorting was performed with a high speed flowcytometer MoFlo SX, (Dakocytomation Fort Collins, CO, USA, equipped with an argon UV-Laser (Coherent Laser®, Inova I 909-6, Dieburg, Germany), set to 200 mW output. Samples were sorted in the presence of Tris-sheath fluid at an average event rate of 25000 cells/sec giving a sorting rate of 3300 cells/sec. Spermatozoa were collected into 10 mL conical plastic tubes (Greiner, Nürtingen, Germany) pre-filled with 500 µL TEST-yolk extender (23). After collection of 8 millions sorted spermatozoa in each collection tube, samples were centrifuged at 840xg for 20 min. The supernatant was discarded and the sperm pellet was resuspended with TRIS egg-yolk freezing extender I and II, then filled into straws and frozen in liquid nitrogen. Time of centrifugation and the temperature of glycerol addition were adjusted according to the experimental design.

Experimental design

Tubes containing sorted spermatozoa were submitted to three different protocols. Sorted sperm samples of the first group (group A) were kept at room temperature until the end of daily sorting (4-6 h after onset of sorting). Centrifugation was performed at the end of this holding period. The sperm pellet was gradually diluted with TRIS freezing extender I and II to a concentration of $20.5x10^6$ spermatozoa/mL. Plastic straws (Fine pailette, 0.25 mL, Minitüb, Tiefenbach, Germany) were filled with 3.3 millions spermatozoa and cooled to 5 °C over a 2 h period. Freezing in the vapour of LN_2 was performed approximately 4 h after glycerol addition (Table 1).

Sorted semen samples in the second group (group B) were processed similarly to samples of group A, except that centrifugation was performed immediately after collection tubes were filled with 8x106 spermatozoa. The difference between group B and C was the addition of TRIS freezing extender II at 5 °C, 45 min before freezing in LN_2 (Table 1). Control samples of unsorted semen were frozen as described for group A.

Table 1: Processing of the sorted spermatozoa

	Centrifugation	Glycerol addition
Group A	4-6 h after onset of sorting	Room temperature
Group B	Immediately after sorting	Room temperature
Group C	Immediately after sorting	5 °C
Control	Unsorted	Room temperature

All sorted semen samples and unsorted controls from one sorting day were frozen at the same time according to the previously described experimental design. Frozen samples were kept in LN_2 and analysed or used for insemination within 2-5 months after freezing. The straws were thawed at 37 °C for 17 sec.

Analysis of frozen thawed samples

Reanalysis of sorting purity

Reanalysis to identify sort purity was performed after thawing. Aliquots of 1 million spermatozoa were taken and diluted to 1 mL with TRIS-sample fluid, supplemented with 20 µl of a 0.8 mM solution of Hoechst 33342 and incubated for 20 min at 37 °C. Thereafter, samples were sonicated and filtered as described before (24). Reanalysis was performed at 60 events/sec. The analysis of purity was performed by a curve fitting model.

Motility analysis

Motility of raw semen samples as well as frozen/thawed semen samples was analysed under a phase-contrast microscope (Olympus BX 60, Hamburg, Germany) equipped with heating plate at 37 °C. Two drops and at least three fields per drop of each sample were evaluated at 100x magnification.

Analysis of sperm morphology

Morphological abnormalities (MAS) and acrosome integrity were analysed under a phase-contrast microscope (Olympus BX 60, Hamburg, Germany) at 1000x magnification after fixation in Hancock's solution. At least 200 spermatozoa were examined per sample. Abnormalities of spermatozoa were divided into damaged acrosomes and morphological abnormal spermatozoa.

Viability and membrane stability of spermatozoa (CFDA/PI)

Samples were prepared by mixing 3.3 µL CFDA (51.04 mM 6-carboxyfluorescein diacetate diluted in dimethyl sulfoxide) and 6.6 µL propidium iodide solution (PI) (mixture of 200 µL of 7.48 mM 3,8-diamino-5-(3-diethylaminopropyl)-6-phenylphenanthridinium iodide methiodide and 400 µL fixative solution: 68 µL of a 37 % formaldehyde solution per 10 mL distilled water) with 10 µL semen sample. Samples were incubated at room temperature in darkness for 10 min. From each sample two drops and at least 200 spermatozoa were analysed under a fluorescence microscope (Olympus BX 60; U-MNIB filter, Hamburg, Germany) at 400 x magnification. Spermatozoa were divided into two groups: viable spermatozoa with intact plasma membrane (CFDA positive and PI negative) and morbid spermatozoa (CFDA positive and PI positive).

Capacitation status of the spermatozoa (FITC-PNA/PI; LPC)

Capacitation status of spermatozoa was assessed with a modified protocol as described by Mcnutt and Killian (25). One Eppendorf cup was filled with 1 mL TRIS-sample fluid and another with 800 µL TRIS-sample fluid and 200 µL LPC solution (500µg L-α-Lysophosphatidylcholine and 1 ml Tris-sample fluid). Tubes were equilibrated for 30 min at 39 °C and then supplemented with 50 µL of semen and incubated for another 10 min at 39 °C. Centrifugation of both tubes was then performed at room temperature at 500xg for 5 min. Supernatant was removed to 100 µL and mixed with the sperm pellet, supplemented with 2 µL FITC-PNA (2 mg FITC-PNA in 2 ml PBS) and 4 µL PI solution (1.50 mM 3,8-diamino-5-(3-diethylaminopropyl)-6-phenylphenanthridinium iodide methiodide and 154 mM NaCl in double distilled water) and incubated another 5 min at 39 °C. The samples were then supplemented with 10 µL paraformaldehyde (1 % in PBS) and analysed immediately. At least 200 spermatozoa in two drops were examined under a fluorescence and phase contrast microscope (Olympus BX 60; U-MNIB filter) at 400 x magnification. Spermatozoa were divided into three groups: viable (PNA-negative, PI-negative), acrosome reacted (PNA-positive) and membrane damaged with intact acrosomes (PNA-negative, PI-positive. Percentage of capacitated spermatozoa was calculated from the difference between the percentage of acrosome reacted spermatozoa before and after addition of LPC.

Artificial insemination

Sorted frozen/thawed spermatozoa of groups A and B were used only for laboratory assessment, whereas spermatozoa of group C and unsorted controls were also used for artificial insemination. Heifers and cows were submitted to routine in-

semination on one farm and were divided into two groups. Animals in the first group were inseminated into the uterine body 12-24 h after onset of natural oestrus. Cows and heifers of the second group were synchronised with GnRH, and PGF-2α (26). In detail, cows received 100 µg GnRH (Depherelin Gonavet Veyx®, Schwarzenborn, Germany) at day -10, further 0.5 mg Cloprostenol (PGF-2α analogue, Essex, München, Germany) at day -3 and again 100 µg GnRH at day -1. Timed artificial inseminations into uterine body were performed 24 h after the second dose of GnRH on day 0. Heifers were synchronised with single injection of 0.5 mg Cloprostenol and animals coming into heat were inseminated 72 h later into uterine body.

Pregnancies were diagnosed 30-60 days after insemination by transrectal ultrasonography (Aloka®; 5 MHz). All pregnant animals were allowed to go to term.

Statistical analyses

Data were analysed with the statistics programme SigmaStat 2.03. Descriptive statistic was used for analyses of means and standard deviations. Laboratory results were tested for normal distribution and then analysed by One-way ANOVA or ANOVA on ranks and Tukey's test. Fertility results were tested with the chi-square test. Results are presented as mean ± standard deviation.

Results

Frozen/thawed semen samples were analysed after incubation at 37 °C for 15 min (Table 2). Percentage of motile spermatozoa was significantly lower in group A compared to groups B, C and control samples. A higher percentage of motile spermatozoa was observed for bull 2 in comparison to bull 1 in unsorted control samples (P= 0.012).

Table 2: Total motility of spermatozoa (%) after thawing

n=12	Group A	Group B	Group C	Control
Bull 1	16.7 ± 14.5[a]	55.0 ± 8.4	65.0 ± 3.0[b]	60.8 ± 6.6[b]
Bull 2	32.9 ± 22.9[a]	57.5 ± 7.6	64.6 ± 5.4[b]	70.0 ± 3.2[b]
Together	24.8 ± 20.5[a]	56.3 ± 7.7[b]	64.8 ± 4.3[b]	65.4 ± 6.9[b]

Values within rows with different superscripts differ (P<0.05).

The semen samples from group C, which were superior to other two groups of sorted spermatozoa, and unsorted control samples, were thawed separately and subjected to a thermo-resistance test at 37 °C. Analysis of motility in frozen/thawed samples, after incubation on 37 °C for 15 min also did not reveal any significant difference between sorted samples and unsorted controls. After further incubation at 37 °C, the motility decreased faster in sorted samples and was significantly higher in control samples after 3 and 6 h of incubation (Figure 1).

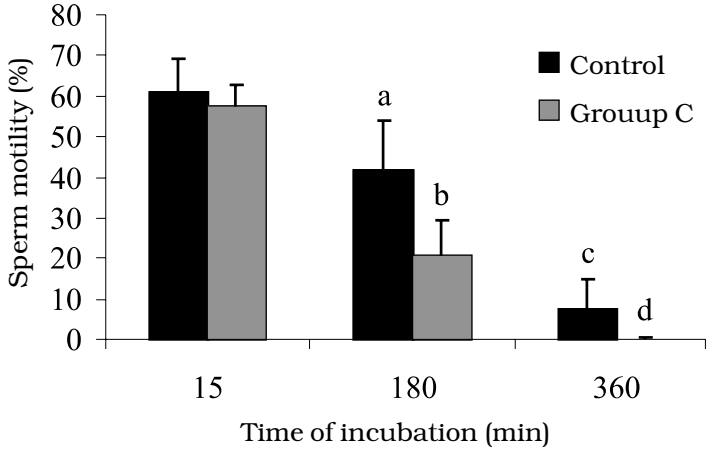

Figure 1: Motility of spermatozoa in frozen/thawed semen samples during incubation over a 6 h (360 min) period at 37 °C (n= 12); Superscripts a and b differ (P<0.001); c and d differ at P<0.05.

Significantly higher percentages of spermatozoa with damaged acrosomes (Table 3) were observed in all groups of sorted spermatozoa compared to unsorted control (P<0.001). The difference between the groups of sorted spermatozoa was not statistical significant. A significant difference between sorted semen and unsorted controls was also observed for the MAS (P<0.001).

Table 3: Percentage of spermatozoa with damaged acrosomes and morphologically abnormal acrosomes (MAS)

(n= 12)	Group A (%)	Group B (%)	Group C (%)	Control (%)
Acrosomes	32.6 ± 7.0[a]	32.0 ± 7.6[a]	29.2 ± 5.3[a]	17.9 ± 7.3[b]
MAS	43.3 ± 7.8[a]	44.0 ± 8.1[a]	37.4 ± 6.8[a]	28.6 ± 7.0[b]

Values with different superscripts differ (P<0.001).

Compared to bull 2, bull 1 had higher percentages of damaged acrosomes in group A (P<0.001) and B (P=0.041) (Table 4) and a higher percentage of MAS in group A (P= 0.009). Bull 1 had significantly higher percentage of damaged acrosomes and MAS in all groups of sorted spermatozoa compared to unsorted semen. For bull 2 this differences were not statistically significant.

Table 4: Differences in the percentage of damaged acrosomes and morphologically abnormal spermatozoa (MAS) between bull 1 (B1) and bull 2 (B2)

(n=6)	Group A (%)	Group B (%)	Group C (%)	Control (%)
Acrosomes (B1)	37.2 ± 5.2[a]	36.3 ± 7.9[a]	29.9 ± 3.7[a]	14.2 ± 5.5[b]
Acrosomes (B2)	28.1 ± 5.7	27.7 ± 4.4	28.4 ± 6.6	21.7 ± 7.4
MAS (B1)	47.3 ± 6.0[a,e]	47.3 ± 8.4[a,e]	38.7 ± 5.7[c,f]	26.0 ± 4.8[b,d]
MAS (B2)	39.3 ± 7.6	40.7 ± 6.9	36.2 ± 7.6	31.2 ± 8.3

Values within rows with superscripts differed a:b (P<0.001); c:d and e:f (P<0.05).

Percentage of viable (CFDA-positive) spermatozoa was higher (P<0.001) in group C (45.7 ± 6.3 %) compared to group A (28.3 ± 4.9 %), B (30.6 ± 8.1%) and control samples (33.5 ± 6.7 %). Percentage of morbid spermatozoa (CFDA and PI positive) was lower (P<0.001) in group C (36.5 ± 5.8 %) and control (35.8 ± 6.3 %) compared to group A (51.4 ± 7.1 %) and B (46.0 ± 8.3 %). Compared to bull 2, bull 1 had lower percentages of viable spermatozoa in all tested groups (P<0.05) and a significantly (P<0.05) higher percentage of morbid spermatozoa in group C. As presented in table 5, both bulls had significantly higher percentages of viable spermatozoa in group C compared to group A, B and control samples. Both bulls had significantly higher percentages of morbid spermatozoa in group A compared to group C and control samples. Bull 2 had also a significantly higher percentage of morbid spermatozoa in group A compared to group B and in group B compared to group C.

Table 5: Difference in the percentage of viable (CFDA+) and morbid (CFDA+/PI+) spermatozoa between bull 1 (B1) and bull 2 (B2)

(n=6)	Group A (%)	Group B (%)	Group C (%)	Control (%)
CFDA+ (B1)	26.0 ± 4.7[a]	25.3 ± 4.7[a]	43.0 ± 4.7[b]	28.7 ± 4.2[a]
CFDA+ (B2)	30.6 ± 4.0[a]	35.9 ± 7.4[a]	48.3 ± 6.7[b,c]	38.2 ± 5.1[d]
CFDA+/PI+ (B1)	50.8 ± 8.7[c]	48.8 ± 9.3	40.0 ± 3.7b[d]	37.0 ± 7.4[d]
CFDA+/PI+ (B2)	52.0 ± 5.6[a,c]	43.2 ± 7.0[d,e]	33.3 ± 5.6[b,f]	34.6 ± 5.5[b]

Values within rows with superscripts differed a:b (P<0.001); c:d and e:f (P<0.05).

A significant higher percentage of capacitated spermatozoa was observed in group A in comparison to both other sorted groups and control. Bull 1 in comparison to bull 2 had lower percentages of capacitated spermatozoa in group B (9.0 ± 3.6 % and 13.8 ± 3.4 %, respectively) and C (9.1 ± 2.6 % and 13.6 ± 5.0 %, respectively).

Table 6: Percentage of capacitated spermatozoa (PNA/LPC-Test)

Group A (%)	Group B (%)	Group C (%)	Control (%)
16.3 ± 4.0[a]	11.4 ± 4.2[b]	11.3 ± 4.5[b]	7.9 ± 4.3[b]

(n= 12) Values with different superscripts differ (P<0.05).

Significantly more pregnant animals were observed after AI with unsorted semen compared to groups with flowcytometrically sorted semen. More pregnancies were achieved in bull 2 compared to bull 1 for the sorted semen (P<0.005) and in heifers than in cows for the unsorted controls (P<0.007).

Table 7: Pregnancy rates and number of animals inseminated in the natural oestrus with flowcytometrically sorted spermatozoa and unsorted controls

	Sorted spermatozoa		Controls	
	(%)	n	(%)	n
Bull 1	12.1[a]	66	57.0[b]	128
Bull 2	26.2[c]	42	55.7[d]	79
Cows	18.8[a]	69	47.3[b]	110
Heifers	15.4[a]	39	67.0[b]	97
total	17.6[a]	108	56.5[b]	207

Values within rows with different superscripts differed a:b (P<0.001); c:d (P<0.05).

Insemination with unsorted semen in animals with synchronized oestrus led to significantly lower pregnancy rates compared to animals inseminated in natural oestrus (P= 0.012). No difference was observed between animals inseminated in synchronised and normal oestrus with sorted spermatozoa (P= 0.629). Overall pregnancy rates in the synchronised animals did not differ between sorted spermatozoa and unsorted controls (P= 0.076). Significantly more pregnancies were observed for cows and bull 1 after insemination with unsorted compared to sex sorted spermatozoa. Sex ratios, as predicted by resort analysis, differed from the 50:50 ratio (P<0.001); 84.5 % of the offspring born were females.

Table 8: Pregnancy rates and number of animals inseminated in the synchronised oestrus with flowcytometrically sorted semen and unsorted controls

	Sorted spermatozoa		Controls	
	(%)	n	(%)	n
Bull 1	12.2[a]	49	38.5[b]	26
Bull 2	32.5	40	34.5	29
Cows	16.9[c]	65	35.7[d]	42
Heifers	33.3	24	38.5	13
total	21.3	89	36.4	55

Values within rows with different superscripts differed a:b (P<0.001); c:d (P<0.05).

Discussion

The goal of this study was to analyse the effects of different modifications in sperm processing after sorting on the quality of frozen/thawed spermatozoa and to investigate the differences in the quality and fertility between sorted and unsorted frozen/thawed semen. Different processing of sorted spermatozoa

had a significant impact on the quality of the frozen/thawed spermatozoa. Post thaw motility of spermatozoa was significantly higher in group B, C and unsorted controls compared to the samples in group A. Further incubation at 37 °C showed significant reduction of motility in sorted samples compared to controls. This may reflect the situation in the female genital tract, and sorted spermatozoa that have been processed especially according to group C, may have little lifetime after AI to reach the oocyte in the oviduct. Therefore, it has been proposed to inseminate closer to the expected time of ovulation and to inseminate deep into the uterine horn in order to avoid sperm losses during the transport through the uterus, and to shorten the time interval between insemination and the passage through the utero-tubal junction.

A reason for the lower quality and the shorter lifespan could be the sorting itself (7). However, as the sorting was identical for groups A, B and C, the post-sorting process also has a major impact on semen quality. The observed reduction of motility in group C may have been caused by reactive oxygen species (ROS) production. Exposure of spermatozoa to higher temperatures and centrifugation are known to increase the production of ROS and consequently lipid peroxidation (13, 27). Removal of seminal plasma by high dilution during sorting promotes the lower resistance of sorted spermatozoa against such damage (11, 28, 29). Increased morphological damage, especially of acrosomes, may also indicate a higher ROS production (30). The percentage of capacitated spermatozoa was calculated from the difference between acrosome reacted spermatozoa before and after LPC treatment. A statistically higher percentage of capacitated spermatozoa was observed in group A in comparison with unsorted control samples. One of the mechanisms for capacitation and the acrosome reaction is the lipid peroxidation of the sperm plasma membrane (30-32). The results indicate that immediate centrifugation and replacement of sorting extender with an extender containing egg yolk, which has an antioxidant ability (16, 33), offered protection against ROS.

Differences between groups B and C are mainly caused by the presence of glycerol at room temperature. It is well known that glycerol is toxic to spermatozoa and therefore should be added to sorted semen shortly before the freezing process starts (19, 34).

Labelling of spermatozoa with CFDA and PI resulted in three distinct populations: green (enzyme active with intact plasma membrane), green/red (enzyme active with damaged plasma membrane), red (dead) (35-37). The most important information in this test is the percentage of viable and membrane intact spermatozoa, represented in the green population. The highest percentages of viable and membrane intact spermatozoa were detected in group C. In groups A and B significantly more cells were damaged; it is also interesting that control samples were more damaged than the sorted sperm of group C. The reason for this result is discarding damaged sperm during sorting. Food dye was added to the spermatozoa before sorting, and only enters into spermatozoa with damaged plasma membranes. This reduces the emission of Hoechst 33342 stain and enables exclusion of damaged sperm during sorting (18).

A statistical significant effect of the bull was found for motility, morphological changes, viability and the percentage of capacitated spermatozoa. These results are in agreement with other studies showing significant bull effect on the quality and the fertilizing capacity of spermatozoa, especially if the spermatozoa are highly diluted (38, 39). Further, they also indicate the importance of bull selection for success of flow-cytometrical sorting (40).

Sperm fertilizing abilities were further tested in the field insemination trial. Pregnancy rates after AI during natural oestrus were lower for sorted spermatozoa (17.6 %) compared to unsorted controls (56.5 %) and were significantly affected by the bull ($P<0.005$). In synchronised animals the difference in pregnancy rates between sorted and unsorted semen dropped to 15 % and pregnancy rates for bull 2 did not differ between sorted samples and unsorted controls.

This results showed a significant effect of the bull on the pregnancy rates after artificial insemination with sex sorted spermatozoa, which is in agreement with other reports indicating high importance of bull selection for insemination with reduced number of spermatozoa (38, 40, 41, 42). Since the differences between the bulls were observed only for the sex sorted and not for the unsorted semen samples, the results also indicate the variability in resistance of spermatozoa to flow-cytometrical sorting between the semen donors. Further, the pregnancy rates in unsynchronised animals were significantly lower in the groups inseminated with sex sorted compared to unsorted semen. The reports from other studies also showed a reduction of pregnancy rates after artificial insemination with sex sorted semen, but mostly in

all studies this reduction of fertility as compared to unsorted semen was within 90% for heifers (4, 43). A high reduction of pregnancy rates after artificial insemination with sex sorted semen in this study could be due to reduced quality of the sorted semen or to management failure. Since the preparation and the quality of the sorted semen used for artificial insemination in this study only differed marginally from these two parameters as reported by other authors (4, 18), it is not likely that this would affect the pregnancy rates to a large extent as obtained in our study. The pregnancy rates in synchronized animals were significantly lower compared to animals inseminated in normal oestrus, but the difference between sorted and unsorted semen was smaller and not significantly different. Lower pregnancy rates in animals with synchronised oestrus could be due to a lack of synchronization in some animals (44). We did not find any significant reduction of overall fertility after artificial insemination of synchronized animals with sorted semen. The reduction of fertility was observed only in bull 1 and in bull 2 fertility was comparable to unsorted semen. These results also indicate the difference between bulls in their resistance to flow-cytometrical sorting. A lower effect of flow-cytometrically sorting on pregnancy rates in synchronised as compared to unsynchronized animals, could be explained by management failure. In our study the animals were inseminated under normal farm conditions and the inseminations with sorted semen were performed at the same time in oestrus as for unsorted semen. Shorter viability of sorted compared to unsorted semen (45, 46) is probably responsible for the reduction of the fertilizing capacity of flow-cytometrically sorted spermatozoa in this study.

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VPLIV LOČEVANJA BIKOVIH SEMENČIC PO SPOLU NA KVALITETO IN OPLODITVENO SPOSOBNOST PO ODMRZOVANJU

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Izvleček: Namen raziskave je bil, ugotoviti vpliv različnih postopkov priprave sortiranega semena pred zamrzovanjem v tekočem dušiku na kakovost in oploditveno sposobnost odmrznjenega semena ter ugotavljanje razlik med sortiranim in nesortiranim semenom. Seme je bilo pridobljeno od dveh bikov črno-bele pasme. Kakovost odmrznjenih vzorcev je bila preiskovana s pomočjo gibljivosti, morfologije, s testom za ugotavljanje stabilnosti membran (6-CFDA/PI) ter kapacitacije (barvanje s pomočjo FITC-PNA/PI z dodatkom L-α-Lysophosphatidylcholine). Podaljšana inkubacija visoko razredčenega sortiranega semena je imela statistično značilen negativni vpliv na gibljivost (P<0,001) in kapacitacijo (P<0,05). Stabilnost celičnih membran je bila statistično značilno (P<0,001) višja pri sortiranih vzorcih, pri katerih je bil glicerol dodan pri 5 °C, v primerjavi z dodatkom glicerola pri sobni temperaturi. Neodvisno od postopka priprave je bil v sortiranem semenu v primerjavi z nesortiranim ugotovljen višji odstotek poškodovanih akrosomov in skupnih morfoloških nepravilnosti (P<0,001). Poleg osnovnih raziskav je bil opravljen tudi termorezistentni test pri 37 °C. V test je bilo vključeno sortirano (centrifugiranje neposredno po sortiranju in dodatek glicerola pri 5 °C) ter nesortirano odmrznjeno seme. Neposredno po tajanju razlika v gibljivosti med sortiranim in nesortiranim semenom ni bila statistično značilna. Po podaljšani inkubaciji pri 37 °C je bil po 3 (P<0,001) in 6 urah ugotovljen statistično značilen nižji (P<0.05) odstotek gibljivih semenčic v sortiranem v primerjavi z nesortiranim semenom. Preiskava in primerjava oploditvene sposobnosti sortiranega in nesortiranega semena je bila opravljena s pomočjo poskusnih osemenitev krav in telic v času normalnih in sinhroniziranih pojatev. Pri osemenitvah med normalnimi pojatvami je bila ugotovljena statistično značilno višja (P<0,001) brejost z nesortiranim semenom (56,5 %) v primerjavi s sortiranim (17,6 %). Pri živalih s sinhroniziranimi pojatvami je bil odstotek brejih živali, osemenjenih z nesortiranim semenom, statistično značilno nižji (p= 0,012) v primerjavi z živalmi, osemenjenimi med naravnimi pojatvami, vendar pa pri tej skupini ni bilo statistično značilne razlike med nesortiranim (36,4 %) in sortiranim semenom (21,3 %). Poleg tega smo ugotovili statistično značilen vpliv bika na oploditveno sposobnost sortiranega semena (P<0.05).

Ključne bsede: sperma - analize; spermatozoa - fiziologija - ultrastruktura; kontrola kvalitete; sperma, konzerviranje; ose - enjevanje, umetno - veterinarsko - metode; brejost; nosečnost, število; biki