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Influence of Sperm Number Per Straw on the Post-Thaw Sperm Viability and Fertility of Swedish Red and White A.I. Bulls

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Januskauskas, A., L. Söderquist, M. G. Håård, M. Ch. Håård, N. Lundeheim and H. Rodriquez-Martinez: Influence of sperm number per straw on the post-thaw sperm viability and fertility of Swedish red and white bulls. Acta vet. scand. 1996, 37, 461-470. – Semen from 5 Swedish Red and White bulls, approved in the fertility and progeny testing programme of a bull center, was split-frozen to produce straws with 15 or 10×10^6 spermatoza each (control and treatment dose, respectively). Post-thaw sperm viability was evaluated by visual assessment of sperm motility (MOT), measurement of ATP (Adeinosin Tri Phosphate) contents by luminometry, assessment of membrane integrity with combined fluorophore probes [Calcein AM (CAM)/Ethidium homodimer (EthD-1)] and by using a hyposmotic swelling test (ORT) The straws were used for a total of 16 651 artificial inseminations (A.I). No statistically significant difference was recorded between the 2 treatments for any of the post-thaw sperm viability parameters. In addition, a significant bull effect was evident for most post-thaw sperm traits assayed. Significant variation in overall fertility (56-days NRR) was recorded among the bulls used. A.I. with a reduced number of spermatozoa (10×10^6/straw) resulted in a 2%-units decrease (n.s.) compared with controls (67.8% ± 4.8%, means ± SD) in overall fertility. In the control split-sample (15×10^6 spermatozoa/straw), MOT did not show any statistically significant correlation with fertility ($r = 0.41$, $p = 0.07$). However, MOT was correlated with the percentage of spermatozoa depicting progressive motility (category A_1, $r = 0.45$, $p<0.05$) as assessed with CAM/EthD-1. The latter was correlated with ATP contents ($r = 0.57$, $p<0.01$), expressed as the percentage of viable spermatozoa. Both CAM/EthD-1 and ATP contents showed a statistically significant correlation with ORT ($r = 0.45$, $p<0.05$ and $r = 0.61$, $p<0$ 05, respectively). In the straws with the reduced sperm number (10×10^6 spermatozoa), post-thaw motility was significantly correlated with fertility ($r = 0.50$, $p<0.05$) and ATP-total contents ($r = 0.48$, $p<0.05$). CAM/EthD-1 and ATP contents (as million viable spermatozoa) were significantly correlated ($r = 0.47$, $p<0.05$). CAM/EthD-1 was significantly correlated with ORT ($r = 0.48$, $p<0.05$). In conclusion, the results indicated that the freezing-thawing of straws with a reduced sperm concentration (10×10^6 spermatozoa) did not alter the post-thaw viability or overall fertility of the bull semen used. However, in view of the significant bull effect found in the limited population studied, we recommend that such a reduction in sperm number/straw be based on the fertility of the bull in question.

motility; ATP; membrane integrity; hypo-osmosis.

Introduction

The optimum number of spermatozoa required to achieve maximum fertilization for the individual bull has yet to be established. Differences between bulls and variation in sperm survival in the female genital tract can be masked by using a high number of spermatozoa *(Shannon & Vishwanath* 1995). Based on their studies, *Uwland* (1984) and *Håård & Håård* (1984) proposed that 15 million spermatozoa per straw be used to obtain optimum fertility rates. This sperm number is nowadays routinely used for artificial insemination (A.I.) in Sweden. By further lowering the sperm number per straw, without a reduction in fertility, the A.I. industry could reduce production costs and wastage by increasing the dose production per bull. Such a reduction in sperm number/straw would require an improvement in our ability to accurately assess the post-thaw number of viable spermatozoa.

Different methods have been developed for evaluating semen quality and predicting fertility in an accurate and objective way *(Amann* 1989). Visual assessment of sperm motility under phase contrast microscopy is the most commonly used method, despite being a rather poor indicator of the fertilizing capacity of a given semen sample *(Amann* 1989). Reports on the assessment of ATP contents and production rates as indicators of sperm viability and predictors of the fertilizing capacity are contradictory *(Foulkes & Mac Donald* 1979, *Chan & Wang* 1987, *Comhaire et al.* 1987, *Söderquist et al.* 1991). Various attempts have also been made to evaluate the integrity of the membrane of the spermatozoa. This work has revealed, for example, that supravital fluorescent markers seem useful in detecting membrane damage following cryopreservation procedures *(Woelder* 1991). Various fluorophore combinations have been successfully used for microscopic assessment of mammalian sperm samples, among those carboxifluorescein diacetate (C-FDA) and propidium iodide (PI, *Harrison & Vickers* 1990, *Ortman & Rodriguez-Martinez* 1994), SYBR-14/PI *(Garner & Johnson* 1995) and Calcein acetylmethyl ester (CAM) and ethidium homodimer (EthD-1) *(Althouse & Hopkins* 1995, *Januskauskas & Rodriguez-Martinez* 1995). The functionality of the sperm membrane under hypo-osmosis has also been used to evaluate human *(Rogers & Parker* 1991), canine *(England & Plummer* 1993) and bovine *(Revell & Mrode* 1994) semen.

The objective of the present investigation was to study the post-thaw sperm viability and fertility of bull spermatozoa used for A.I., when reducing the concentration from 15 to 10 million spermatozoa/straw in a split-sample trial. Sperm viability was measured by visual assessment of sperm motility, measurement of ATP contents by luminometry (SVT® system), evaluation of membrane integrity with combined fluorophore probes (CAM/EthD-1) and by using a hypo-osmotic swelling test (ORT).

Materials and methods

Animals

Deep-frozen semen from 5 Swedish Red and White bulls (aged between 6 and 6.5 years) was evaluated in this study. The bulls had a history of normal breeding performance and were used routinely at the bull station (Svensk Avel, Skara) for commercial A.I. after having been approved in the fertility and progeny testing programme. They did not present clinical abnormalities; their libido was normal, and their fresh semen spermiogrammes were within acceptable physiological limits, i.e. more than 85% morphologically normal spermatozoa, at least 70% motile and a total sperm number above 3×10^9.

Semen collection and processing

Two consecutive ejaculates were collected with only a few minutes rest in between. The con-

centration of each ejaculate was determined with a photometer. After subjective motility evaluation, the ejaculates were pooled and diluted with a commercial extender (Triladyl®, Minitüb, Germany) to a concentration of 138×10^6 spermatozoa/ml. The diluted semen was cooled to ambient temperature during 20 min in a room-tempered water bath. The semen was then divided into 2 parts that contained ⅖ and ⅗ of the total volume. One part was extended with Triladyl® to a final concentration of 45×10^6 spermatozoa/ml, and the other part was extended to a final concentration of 69×10^6 spermatozoa/ml. This final dilution was made in one step at ambient temperature. The semen was filled in 0.25-ml plastic straws, resulting in A.I. doses containing a total of 15×10^6 and 10×10^6 spermatozoa, respectively. The straws were put on racks and transferred to a cold cabinet (4°C) for a 4 h long equilibration. The semen was frozen in liquid nitrogen (LN_2) vapour in a programmable freezer (Digitcool 5300®, IMV, France). The 2 concentrations of the original split sample, were identified with individual, consecutive batch numbers and regarded as a pair. The frozen straws were stored in LN_2 until thawing, which was carried out by immersing them in water at 35°C for 12 sec. Post-thaw motility (MOT) was determined subjectively at the bull station, in the semen from 2 straws/-batch, under a microscope equipped with phase-contrast optics and a warming stage (38°C). Only semen with a minimum of 50% MOT was approved for A.I. Sperm concentration was calculated in a hemocytometer (Bürker chamber, *Bane* 1952).

Assessment of ATP content

The ATP content was measured in the semen from 2 pooled straws/batch, using a bioluminescence technique with the FireZyme Sperm Viability Test® (SVT® (FireZyme Diagnostics Inc., Halifax, Nova Scotia, Canada, *Januskauskas & Rodriguez-Martinez* 1995). By this method, 2 aliquots from a semen sample were tested: one is used to determine the total amount of ATP (Total) and the second was used to determine the amount of free ATP outside competent cells in the seminal fluid (Blank). Reagent (Luciferin-luciferase solution, buffers) preparation, internal calibration and testing procedures were performed as indicated by the manufacturers. The reading from the luminometer was used to determine the samples ATP content (Total and Blank ATP, in μg/ml) based on a computer-generated standard curve. The number of viable spermatozoa (in millions/ml) were also calculated by the instrument according to the following formula:

$$\text{Million viable spermatozoa/ml} = \frac{\text{ATP Total} - \text{ATP Blank}}{0.15\ (\mu\text{g ATP per million viable spermatozoa})^*}$$

The number of viable spermatozoa were manually re-calculated to give the percentage of viable spermatozoa based on the hemocytometric estimation of sperm concentration.

Assessment of sperm membrane integrity

Sperm membrane integrity was assessed, in the semen from the same straws/batch, using 2 techniques:

The first method has previously been described by *Januskauskas & Rodriguez-Martinez* (1995). In brief, 20 μl of frozen/thawed semen was incubated in 20 μl of staining medium containing Ethidium homodimer (EthD-1) and Calcein AM (CAM, Molecular Probes Inc., Oregon USA) in 1 ml of Phosphate Buffer Solution (PBS) for 15-30 min at 35°C. Subsamples (5 μl) of the stained suspension were

* according to the setting of the SVT®-instrument.

placed on clean microscope slides and overlaid carefully with coverslips. Random fields were observed with epifluorescence UV-illumination (Diaplan Leitz microscope, equipped with a warm stage (37°C, 400×). For quantification of sperm membrane integrity, 100 cells were counted in each of two 5 μl aliquots from a stained sample. The counted sperm cells were classified as A) having an intact plasmalemma when stained green with CAM and remained unstained with EthD-1 (they were further subdivided into categories A_1 (motile) and A_2 (immotile); B) having a damaged plasmalemma, but an intact acrosome that stained green with CAM and a post-acrosomal region that stained red with EthD-1; and C) having both a damaged plasmalemma and a damaged acrosomal membrane, with the cells remaining unstained with CAM but staining red with EthD-1.

The second method was a slight modification of the osmotic resistance test (ORT) described by *Revell & Mrode,* (1994). Semen (10 μl) was incubated in darkness for 30 min in 1 ml of a 100 mOsm kg^{-1} hypo-osmotic test solution at 35°C, subsequently adding 20 μl of staining solution, containing 4 μl of EthD-1 and 10 μl of CAM in 1 ml of Phosphate Buffer Solution (PBS), and prolonging the incubation time by additional 15 min. Two counts of 100 spermatozoa each were made from each preparation and estimated using the same microscopic equipment as cited above. Spermatozoa were divided into 3 groups. The first group consisted of spermatozoa having intact membranes and fluorescing green throughout their length. They were swollen and some of them depicted residual activity [A]. The second group [B] of spermatozoa had damaged plasma membrane. They were unswollen and stained red at the post-acrosomal and tail regions, whereas the acrosomal region was intact and therefore stained green. The last category of spermatozoa comprised those whose acrosome and plasma membranes were both compromised and therefore stained red and were unswollen [C].

Distribution of fertility measures

To avoid confounding fertility results, the A.I.-doses were mixed and randomly distributed among as many A.I.-technicians as possible to obtain a widespread distribution among herds. From 2 to 5 pairs of batches from each bull were used for inseminations. The percentage reported as not having returned to service within 56 days was used as a measure of fertility of individual batches. Non-return rates (NRR) from the normal A.I. routine were obtained from 42 individual batches used for a total of 16651 inseminations (8410 for 15 million sperm and 8241 for 10 million sperm/doses). The average number of A.I. per batch in each pair was 396.5. The straws were distributed simultaneously, so that the 2 corresponding batches were used randomly and at the same subcenters, thus enabling a direct comparison of the results. The A.I.-technicians were unaware of the trial.

Statistical analysis

Altogether, the data consisted of 42 observations (= batches). The data were subjected to analysis of variance using the GLM procedure *(SAS Institute Inc.,* 1994). The statistical model included the effects of *bull* (5), *concentration* (2) and the *bull * concentration* interaction. Correlation analysis (Spearman correlation coefficients) was used to assess the relationship between semen characteristics within concentration and the relationships between these characteristics and fertility (56 days NRR). Levels of significance in the tables are indicated as follows: $p<0.05$ = *; $p<0.01$ = **; $p<0.001$ = ***. Differences were considered significant when the probability value for equality between 2 least-squares means was less than 5%.

Results

Post-thaw motility in the straws with 15×10^6 spermatozoa (control concentration) did not show a statistically significant correlation with fertility ($r = 0.41$, $p = 0.07$). Post-thaw motility was not significantly correlated with ATP contents (total; $r = 0.30$, $p>0.05$) but it was significantly correlated with the percentage of spermatozoa depicting progressive motility (category A_1, $r = 0.45$, $p<0.05$) when assessed with the combined fluorescent dyes CAM/EthD-1. The incidence of spermatozoa with intact plasmalemma and motile (A_1) was correlated with ATP contents, expressed as the percentage of viable spermatozoa ($r = 0.57$, $p<0.01$). Furthermore, both CAM/EthD-1 and ATP contents showed a statistically significant correlation with ORT ($r = 0.45$, $p<0.05$ and $r = 0.61$, $p<0.05$, respectively).

When correlation analyses were run with data from the A.I. with the lower sperm number (10×10^6 spermatozoa/straw), post-thaw motility was significantly correlated with the 56-day NRR ($r = 0.50$, $p<0.05$) and with ATP contents (total; $r = 0.48$, $p<0.05$). CAM/EthD-1 and ATP contents (as million viable spermatozoa) were significantly correlated ($r = 0.47$, $p<0.05$). CAM/EthD-1 was significantly correlated with ORT ($r = 0.48$, $p<0.05$). No significant difference was recorded for post-thaw sperm viability parameters between the 2 A.I. dose levels used (Table 1). A significant bull effect was evident for most post-thaw sperm traits assayed ($p<0.05$-0.001, Table 3).

The average overall 56 days-NRR for the bulls used was 66.6 ± 5.5 % (means ± SD). The overall difference in NRR between the split-sample batches assayed was 2 %-units (not significant, Table 1), with significant ($p<0.01$) variation among bulls (Table 3). However, the interaction between bull and concentration had no significant effect (Table 3). Three out of the 5 bulls had a lower (n.s.) NRR after A.I. with 10 million spermatozoa/straw, while the other 2 bulls maintained their fertility at the same level as the control A.I.-dose (15 million spermatozoa/-straw, Table 2). One particular animal (bull C) appeared to be the major reason for the variation between bulls; his NRR was significantly lower than those of the other 4 bulls ($p<0.01$, obtained from difference in LS-mean for bull * concentration) for both concentrations assayed (Table 2).

Discussion

It has been established that bull fertility depends on the interactions between both qualitative and quantitative parameters of spermatozoa *(Pace* 1980). The dose-effect relationship for fertile bulls is marked by a limiting factor: beneath a certain threshold level of sperm concentration, which varies between individuals, the probability of fertilization drops abruptly *(Schwartz et al.* 1981). We found that a reduction of the sperm number/straw from 15×10^6 to 10×10^6 spermatozoa resulted in an overall decline in a non-significant non-return rate by 2 percentage units. Individual differences between bulls became more apparent following a reduction of the total sperm number per insemination dose. The number of bulls studied here, however, was too small to allow us to conclude whether a significant relationship exists between the fertility of a given bull at 15×10^6 spermatozoa/straw and its ability to maintain high A.I.-fertility following a decrease in sperm concentration.

The assessment of sperm viability after freezing/thawing made in the present study showed that the dilution level used did not markedly reduce motility, membrane integrity or ATP-contents. Assessment of the post-thaw percent of motile spermatozoa is the most commonly used method for evaluating semen. Significant correlations have been found between motility and

Table 1. Post-thaw sperm viability and fertility for the split concentrations (means ± S.D.).

Viability and fertility	Sperm concentration/straw	
	15 million (n = 21)	10 million (n = 21)
Motility (%)	54.3 ± 3.8	53.6 ± 3.7
ATP (%)[1]	60.8 ± 11.5	64.9 ± 11.2
Membrane integrity (%)[2] A	62.9 ± 12.1	64.1 ± 8.7
Membrane integrity (%)[3] A_1	51.0 ± 12.3	52.3 ± 9.3
Membrane integrity (%)[4] ORT	47.4 ± 19.0	40.8 ± 13.7
Fertility (%) (56-day NRR)[5]	67.8 ± 4.8	65.5 ± 6.0

1) Percentage of viable spermatozoa recalculated from the number of viable spermatozoa per ml given by the SVT® instrument.
2) Percentage of both motile and immotile spermatozoa with intact plasmalemma, assessed by Calceine AM/EtgD-1.
3) Percentage af motile spermatozoa with intact plasmalemma, assessed by Calceine AM/EthD-1.
4) Percentage of spermatozoa with intact plasmalemma, assessed by the modified ORT method.
5) The number of inseminations accounted 8 410 and 8 241 using 15 million respective 10 million spermatozoa per straw.

Table 2. Individual bull fertility (56 days NRR) and the difference in % for the sperm concentrations (split sample, means ± S.D.).

Sperm concentrations ($\times 10^6$)	Bull A	Bull B	Bull C	Bull D	Bull E
15	67.7 ± 3.2	67.6 ± 2.3	61.7 ± 6.7a	68.6 ± 5.3	71.5 ± 2.6
10	68.3 ± 7.0	68.6 ± 3.0	57.0 ± 4.4b	65.2 ± 5.7	68.0 ± 3.2
Difference in %	+ 0.6	+ 1.0	− 4.7	− 3.4	− 3.5

a–b) Difference between bull C and the other bulls within concentration (a = $p<0.05$–0.01, b = $p<0.01$).

non-return rates in some studies *(Lindford et al.* 1976, *Kjæstad et al.* 1993, *Mickelsen & Memon* 1993) but not in others *(Graham et al.* 1980, *Söderquist et al.* 1991). In the present study, a statistically significant correlation was found (r = 0.50), but only for the concentration of 10×10^6 spermatozoa/straw.

Viable cells are the only ones able to maintain a balance between ATP production and consumption. Furthermore, a significant correlation between post-thaw motility and ATP contents has been shown *(Foulkes & Mac Donald* 1979, *Söderquist et al.* 1991, *Januskauskas & Rodriguez-Martinez* 1995). A similar relationship was also found in the present study, but it was only statistically significant for the semen batch with the reduced sperm number (10 million spermatozoa/straw).

Only viable spermatozoa are able to normally interact with the oocyte and pursue fertilization. Thus, it could be expected that the amount of ATP/spermatozoa would be correlated with fertility. However, this relationship between ATP content and fertility has yet to be established. The lack of any correlation between semen ATP content and in vitro fertilization has been reported for human oocytes *(Chan et al.* 1990). A significant positive correlation between ATP content and fertility for bull semen was obtained by *Wood et al.* (1986),

Table 3. Levels of significance for the effects included in the statistical model on some of the analysed traits for frozen-thawed semen.

Source of variation	Degrees of freedom	56-day NRR	Motility %	ATP content (SVT®)			Membrane integrity						
							CalceineAM/Ethidium homodimer				ORT		
				Total µg/ml	Blank µg/ml	Million viable spermotozoa /ml	A1	A2	B	C	A	B	C
Bull	4	**	*	**	**	**	ns	***	***	***	**	ns	**
Sperm concentration	1	ns	ns	***	**	***	ns	ns	ns	ns	ns	ns	ns
Interaction between bull and concentration	4	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

*: $p<0.05$. **: $p<0.01$. ***: $p<0.001$. ns = not significant.

whereas *Söderquist et al.* (1991) demonstrated a negative relationship between ATP concentration and fertility. In the present study, the correlation between ATP contents and fertility was positive, however, not statistically significant for either the 10 or 15 million spermatozoa/-straw, probably because the number of observations assayed was limited. In fact, when the results from both dosages were pooled, thereby doubling the number of observations, the correlation with fertility was statistically significant. The cell membrane plays a crucial role in the survival of the spermatozoa throughout the procedures involved in semen cryopreservation. Spermatozoa with damaged cell membranes cannot attain ion and co-factor concentrations essential for flagellar movement *(De Leeuw et al.* 1991). Therefore it can be speculated that post-thaw motility, ATP content and membrane integrity assessed with different techniques ought to be correlated with each other. Indeed, we observed that ATP content (expressed as percentage of viable spermatozoa) was significantly correlated with the frequency of spermatozoa having intact membranes (A) and depicting motility (A_1) as monitored with CAM/EthD-1, independently of the sperm concentration. There was good agreement between the percentage of viable spermatozoa assessed by SVT® and corresponding values obtained using the supravital staining technique and through the subjective estimation of motility.

The lack of any statistically significant correlations between the values obtained using methods for assessing membrane integrity (CAM/EthD-1 and ORT) and those obtained using methods for assessing fertility seems to have been caused by the variation between bulls, which was observed for both sperm concentrations used. Another contributing factor could have been that the range of values obtained with the ORT tends to be large, as was the case in our study (11-84%) and in the study by *Revell & Mrode* (1994; 11-78%). Many studies have been attempted to find such a relationship between the hypo-osmotic swelling test and fertility, with contradictory results *(Check et al.* 1988, *Rogers & Parker* 1991, *Rev-*

ell & Mrode 1994). The value of the assessment with supravital fluorescent dyes in predicting the fertility outcome needs to be further studied.

In conclusion, a reduction of the sperm number/straw from 15 million to 10 million spermatozoa did not have a statistically significant influence on the post-thaw sperm viability parameters assayed or on fertility results (56-day NRR). However, the influence of sperm number/straw seems to vary depending on the sperm characteristics and fertility level of bulls used.

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Sammanfattning

Påverkan av spermieantalet per strån på uptinings viabilitet och fertilitet hos SRB-semin tjurar

Sperma från 5 avkommebedömda semintjurar (SRB) frystes med sk split sample teknik så att strån innehållande totalt 15 (kontroll) respektive 10 miljoner erhölls Spermiernas livsduglighet undersöktes efter frysning/upptining genom bedömning av. spermiernas motilitet (MOT), ATP innehåll (med hjälp av en luminometer), membran integritet (med hjälp av en kombination av fluorescerande markörer [Calcein AM (CAM)/Ethidium homodimer (EthD-1] och genom användandet av ett sk. hypoosmotiskt swellingtest (ORT). Stråna användes till totalt 16 651 inseminationer Ingen statistiskt signifikant skillnad, vad gäller de undersökta parametrarna efter upptining, kunde noteras mellan de 2 olika spermiekoncentrationerna Vidare sågs en signifikant tjureffekt för de flesta undersökta spermieegenskaper En signifikant variation i fertiliteten (56 dagars NR) kunde ses bland de använda tjurarna Insemination med ett reducerat antal spermier per strå (10 miljoner) resulterade i en sänkning av fertiliteten med 2%-enheter (inte signifikant) jämfört med kontrollen (67,8% ± 4,8%, medelvärde ± SD) Fertilitetsresultatet efter insemination med strån innehållande 15 miljoner spermier uppvisade inget statisiskt signifikant samband med fertiliteten ($r = 0,41$, $p = 0,07$). Emellertid sågs ett samband mellan MOT och den procentuella andelen spermier som uppvisade en progressiv motilitet (kategori A_1, $r = 0,45$, $p<0,05$) vid bedömning med hjälp av CAM/EthD-1. Den senare var vidare korrelerad med ATP innehållet ($r = 0,57$, $p<0,01$) uttryckt som procent viabla spermier. Både CAM/EthD-1 och ATP innehållet uppvisade ett statistiskt signifikant samband med ORT ($r = 0,45$, $p<0,05$ repektive $r = 0,61$, $p<0,05$). Strån med reducerat antal spermier (10 miljoner) uppvisade ett signifikant samband med fertiliteten ($r = 0,50$, $p<0,05$) och ATP-innehållet ($r = 0,48$, $p<0,05$). Vidare var CAM/EthD-1 och ATP-innhållet (uttryckt som miljoner viabla spermier) signifikant korrelerade ($r = 0,47$, $p<0,05$). CAM/EthD-1 uppvisade också ett samband med ORT ($r = 0,48$, $p<0,05$). Sammanfattningsvis tyder reultaten på att frysning/upptining av strån med en reducerad spermiekoncentration (10 miljoner spermier) inte verkar spermiernas livsduglighet eller fertiliteten hos den använda tjursperman.

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