Theriogenology 84 (2015) 413–420

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Use of immobilized cryopreserved bovine semen in a blind artificial insemination trial

Fride Berg Standerholen [a,b], Karin Elisabeth Waterhouse [c], Anne Guro Larsgard [d], Randi Therese Garmo [c,1], Frøydis Deinboll Myromslien [b], Jan Sunde [d,2], Erik Ropstad [a], Geir Klinkenberg [e], Elisabeth Kommisrud [b,c,*]

[a] Department of Production Animal Clinical Sciences, Norwegian University of Life Sciences, Oslo, Norway
[b] Department of Natural Sciences and Technology, Hedmark University College, Hamar, Norway
[c] SpermVital AS, Hamar, Norway
[d] Geno SA, Hamar, Norway
[e] SINTEF Materials and Chemistry, Department of Biotechnology, Trondheim, Norway

ARTICLE INFO

Article history:
Received 30 January 2015
Received in revised form 24 March 2015
Accepted 26 March 2015

Keywords:
Alginate gel
Bull semen preservation
Spermatozoon
Immobilization
Nonreturn rate
Flow cytometry

ABSTRACT

To make timing of artificial insemination (AI) relative to ovulation less critical, methods for prolonging shelf life of spermatozoa *in vivo* after AI have been attempted to be developed. Encapsulation of sperm cells is a documented technology, and recently, a technology in which sperm cells are embedded in alginate gel has been introduced and commercialized. In this study, standard processed semen with the Biladyl extender (control) was compared with semen processed by sperm immobilization technology developed by SpermVital AS in a blind field trial. Moreover, *in vitro* acrosome and plasma membrane integrity was assessed and compared with AI fertility data for possible correlation. Semen from 16 Norwegian Red young bulls with unknown fertility was collected and processed after splitting the semen in two aliquots. These aliquots were processed with the standard Biladyl extender or the SpermVital extender to a final number of 12×10^6 and 25×10^6 spermatozoa/dose, respectively. In total, 2000 semen doses were produced from each bull, divided equally by treatment. Artificial insemination doses were set up to design a blinded AI regime; 5 + 5 straws from each extender within ejaculates in ten-straw goblets were distributed to AI technicians and veterinarians all over Norway. Outcomes of the inseminations were measured as 56-day nonreturn rate (NRR). Postthaw sperm quality was assessed by flow cytometry using propidium iodide and Alexa 488–conjugated peanut agglutinin to assess the proportion of plasma membrane and acrosome-intact sperm cells, respectively. In total, data from 14,125 first inseminations performed over a 12-month period, 7081 with Biladyl and 7044 with SpermVital semen, were used in the statistical analyses. There was no significant difference in 56-day NRR for the two semen categories, overall NRR being 72.5% and 72.7% for Biladyl and SpermVital, respectively. The flow cytometric results revealed a significant higher level of acrosome-intact live spermatozoa in Biladyl-processed semen compared to SpermVital semen. The results indicate that the level of acrosome-intact live spermatozoa in the AI dose did not affect the 56-day NRR for the two semen processing methods. In conclusion, this study has showed that immobilized spermatozoa provide equal fertility results as standard processed semen when AI is performed in a blinded field trial, although the immobilization

* Corresponding author. Tel.: +47 62517247; fax: +47 62517601.
E-mail address: elisabeth.kommisrud@hihm.no (E. Kommisrud).
[1] Present address: TINE SA, Ås, Norway.
[2] Present address: Cryogenetics AS, Hamar, Norway.

http://dx.doi.org/10.1016/j.theriogenology.2015.03.028

procedure caused increased sperm damage evaluated *in vitro* compared to standard semen processing procedure.

1. Introduction

Artificial insemination (AI) is commonly used for a range of domestic animals and in aquaculture. The technique can be applied with fresh, liquid, and cryopreserved semen, depending on species, and is the most valuable tool for animal breeding programs and genetic improvements [1]. In commercial animal production, fertility results are crucial to the total economic outcome. In the cattle breeding industry, AI is mainly applied with cryopreserved spermatozoa, a technology developed around 1950 on the basis of the discovery of glycerol being protective to sperm cells during freezing [2]. To obtain good fertility results, AI must be performed within a restricted number of hours before ovulation. Therefore, new technologies that prolong the lifetime of spermatozoa *in vivo* after AI will be beneficial to the industry because timing of AI relative to ovulation will be less critical.

Alginate gels are formed by interactions between divalent ions such as Ca^{2+} and block structures of the guluronic acid in the alginate polymer chain. Therefore, the formation of alginate gels can be conducted under very mild conditions, and have thus been commonly used for immobilization of various types of cells [3,4]. However, immobilization in alginate has been most commonly used as a starting point for later formation of various types of capsules with a liquid core. Several studies of encapsulation of spermatozoa within microcapsules are published [5–9]. These studies have used methods resulting in particles where the spermatozoa are located within a liquid core surrounded by a membrane.

Nebel et al. [5] reported that encapsulation was compatible with bovine sperm survival and that negligible sperm injury was observed during storage of encapsulated spermatozoa at 37 °C. Later, several studies have reported that encapsulated spermatozoa maintain their fertilizing capacity after AI [6–8], also when AIs were performed in proestrus, i.e., early relative to ovulation [9]. The technique has been applied with semen from other species such as ram [10] and boar [11], and finally, novel systems for encapsulation and release of bovine spermatozoa in capsules with a liquid core have been described [12,13].

The SpermVital technology for immobilization and cryoconservation of bull spermatozoa for AI has been developed during the past decade [14] and is implemented in large-scale production. This technology uses a fundamentally different approach to immobilize spermatozoa than previously published methods as spermatozoa are immobilized within a solid gel network made of calcium alginate gel. Immobilization within a solid gel network may have advantages compared to encapsulation within capsules. Using this method, movement of the immobilized spermatozoa might be restricted because of constraints of the gel network. This might resemble the situation in cauda epididymis where physical limitation of movement due to high concentrations of cells and presence of high viscosity polymers may be one of the factors that influence survival and maintenance of functionality of spermatozoa over long periods of time [15].

The success of bovine AI programs, regardless of semen processing techniques, is largely dependent on the use of good-quality semen, and it is reported that sperm characteristics are correlated to differences in fertility [16]. Simultaneously evaluating postthaw viability and acrosome integrity of spermatozoa by flow cytometry is a valuable testing tool in both research and routine work [17].

The SpermVital technology has been shown to be applicable for AIs performed early relative to ovulation (unpublished data); however, it is also important to know how the semen performs when used at normal timing of AI. The dissolving of the gel and thereby release of sperm cells is designed to last for at least 24 hours. Insemination performed at conventional timing may therefore be suboptimal for SpermVital-processed semen because fewer sperm cells are assumed to be available at the time of ovulation. The question to be raised in this investigation was whether enough sperm cells are released and capable of fertilization at conventional timing of AI.

The aim of this study was to compare the fertilizing capacity of semen processed with two different methods: conventional (Biladyl) and immobilization processing (the first-generation SpermVital technology) [14] in a blinded field trial. Furthermore, the semen samples were analyzed to reveal differences in sperm quality between the processing techniques by assessing acrosome and plasma membrane integrity. Moreover, the association between these sperm quality parameters and field fertility was determined.

2. Materials and methods

2.1. Semen samples for insemination trial and in vitro studies

All procedures for semen processing, being in compliance with European Union Directive 88/407, were approved by the Norwegian Food Safety Authority. The insemination trial was not in violation of any ethical guidelines or legislation.

During the June–August 2010 period, semen from 16 Norwegian Red (NRF) young bulls with unknown fertility was collected and processed by the breeding company Geno SA (Geno Breeding and AI Association, Hamar, Norway). The bulls had a mean age of 469 days (range, 432–497) on the first day of semen collection. Collection was performed once a week, with two separate ejaculates collected with approximately 15 minutes of interval before pooling. Collection continued until a minimum of 2000 doses were produced from each bull, resulting in processing of 4 to 9 ejaculates per bull, in total 85 ejaculates. Sperm concentration was estimated by spectrophotometer, and motility was evaluated by phase-contrast microscopy before and

after cryopreservation. Semen having a sperm concentration below 390 million/mL was discarded.

All ejaculates were split in two aliquots ("split-sample" design), one control processed routinely with the Biladyl (egg yolk tris) extender (13500/0004-0006; Minitube GmbH, Tiefenbach, Germany) and the other processed by the experimental first-generation SpermVital extender (SpermVital AS, Hamar, Norway), later referred to as B and SV, respectively. The two aliquot volumes were adjusted to achieve equal number of semen doses with each extender, and they were given a unique batch number for straw printing and later identification of results from AIs. The semen aliquots were diluted in a two-step procedure, with B extender to a final concentration of 12×10^6 spermatozoa/-dose and with SV extender to a final concentration of 25×10^6 spermatozoa/dose. After dilution, semen was filled into white, translucent, 0.25-mL Top Bull Straws (IMV 017011; IMV Technologies, L'Aigle, France) and cryopreserved according to standard procedures for B and SV processing. Semen doses were then stored in liquid nitrogen at −196 °C until finalizing quality control, quarantine, and consecutive distribution. Only ejaculates with postthaw motility of at least 50% in both B- and SV-extended aliquots were used in the trials, resulting in exclusion of one ejaculate from each of three bulls. The trial was blinded, with processing methods applied being unidentifiable to the AI personnel. Straws (5 B + 5 SV) from each ejaculate were packed in ten-straw goblets and distributed to AI technicians and veterinarians all over Norway for the field trial. In total, 170 batches of semen were distributed (85 B and 85 SV). Accordingly, doses from all batches, separated and identified by goblets, were shipped to the laboratory for *in vitro* experiments.

In addition, semen doses from three bulls were produced for a control *in vitro* experiment performed to evaluate the possible influence of the difference in final sperm concentration between the two processing methods on outcome of sperm quality analyses. Biladyl and SV semen were produced by split-sample of each ejaculate with 12×10^6 and 25×10^6 spermatozoa/straw for both semen extenders, totally four batches per bull.

2.2. Staining of frozen–thawed sperm samples for flow cytometric analyses

Propidium Iodide (PI, P4864; Sigma–Aldrich, St. Louis, MO, USA) was used to discriminate between live and dead spermatozoa, i.e., plasma membrane intact and degenerated, respectively. Lectin peanut agglutinin (PNA) from *Arachis hypogaea* (peanut) conjugated with Alexa Fluor 488 (PNA–Alexa 488, L21409; Invitrogen, Paisley, UK) was used to identify the proportion of acrosome-intact and reacted or degenerated spermatozoa, whereas MitoTracker Orange CMTMRos (MO, M7510; Invitrogen) was used as a sperm identification probe. Stock solutions of all fluorochromes were prepared in DMSO (D-5879, Sigma–Aldrich) according to product information. A PBS staining solution of fluorochromes with stock concentration of 0.05 mg/mL PNA–Alexa 488, 1.5 mM PI, and 10 μM MO was prepared. For semen analyses, semen doses were thawed for 1 minute in a water bath at 37 °C. Thereafter, to equalize sperm concentration, 250 and 500 μL gel-dissolving medium (provided by SpermVital AS) prewarmed to 37 °C was added to the B and SV semen, respectively. Samples were placed on a tilt tray for 5 minutes in an incubator at 37 °C before further incubation for 3 hours at 37 °C. After incubation, the samples were transferred to a heating block at 37 °C, and 30 μL semen was added to 470 μL prewarmed staining solution to a final concentration of ~2 million sperm cells/mL and a final concentration of staining solution of 50 ρg/μL of PNA–Alexa 488, 7.48 μM PI, and 0.15 μM MO. Samples were then incubated on the heat block at 37 °C for 10 minutes before flow cytometric analysis. Semen from three straws per batch were stained and analyzed in two replicates by flow cytometry.

2.3. Flow cytometric assessment

Measurements were performed on a Cell Lab Quanta SC MPL flow cytometer (Beckman Coulter, Fullerton, CA, USA), later referred to as Quanta. The flow cytometry–generated data were analyzed in Cell Lab Quanta SC MPL Analysis software program (Beckman Coulter) and then exported to Microsoft Office Excel (Microsoft, Redmond, WA, USA) for statistical analysis. The instrument was checked daily for optical alignment by running Flow-Check beads (6605359; Beckman Coulter). An unstained sperm sample was included as the negative control. Electronic volume and side scatter signals were used to identify spermatozoa, whereas MO was used as an additional sperm identification probe. The three dyes were excited using a 488 nm argon laser. Peanut agglutinin–Alexa 488 and MO fluorescence emission was detected using a 510 to 540 nm band pass filter (FL1) and a 560 to 590 nm band pass filter (FL2), respectively, whereas PI fluorescence was detected using a 670 nm long pass filter (FL3). Compensation was performed before collection of data with unstained spermatozoa and spermatozoa stained singularly with PNA–Alexa 488, MO, and PI. The regions were set and gating was performed as described by Standerholen et al. [18] to exclude debris particles and reveal percentages of (1) acrosome-intact dead (AID) spermatozoa, (2) acrosome-reacted dead (ARD) spermatozoa, (3) acrosome-intact live (AIL) spermatozoa, and (4) acrosome-reacted live (ARL) spermatozoa.

2.4. Statistical analyses

Data on 56-day nonreturn rate (NRR) for the first inseminations were compared for B and SV semen. Animals with inseminations repeated within 3 days after the first AI were excluded from the analyses. The possible effects on NRR were analyzed by the General Linear Models Procedure software, using Statistical Analysis System (SAS) version 9.3 (SAS 2002–2010) for Microsoft Windows (SAS Institute Inc., Cary, NC, USA).

Least-square analysis was used to estimate the effect of bull or batch, month of AI, parity of the females, and semen processing on NRR by the following model:

$$Y_{ijklmn} = \mu + l_i + p_k + t_l + v_m + e_{jklmn}$$

where:

Y_{ijklmn} = observation per heifer/cow;
μ = overall mean;

l_i = effect of bull, i = 1 to 16 or l_i = effect of batch, i = 1 to 166;
p_k = effect of month of AI, k = 1 to 12;
t_l = effect of parity, l = 0 to 4;
v_m = effect of semen processing, m = 1,2;
e_{jklmn} = random error.

Statistical analyses of the flow cytometry–generated postthaw sperm quality were performed using JMP software, version 8 for Microsoft Windows (SAS Institute, Inc.). Percentages of AID, ARD, AIL, and ARL spermatozoa are presented as mean (standard deviation) and by 95% confidence interval (CI) plot within the semen extender. Variation in the proportion of spermatozoa that potentially may fertilize after AI, AIL (%), were compared and tested with nested (hierarchy) ANOVA (bull, straw, replicate, processing) to reveal the contributed variation by the explanatory variables to the total variation explained by the statistical model.

Because of the difference in final sperm concentration of B and SV semen, the percent AIL data were transformed to millions of AIL spermatozoa per dose (million/straw) before testing a possible effect of the proportion of the potential fertilizing spermatozoa postthaw on outcome of AI. A paired t test was used to make paired comparisons of differences in AIL spermatozoa (million/straw) between B and SV semen samples within ejaculate and bull. Results from this test were described by 95% CI and presented in a CI plot. If the CI in the plot contained the reference line at zero, then the means of the two samples, SV and B, from the same bull were not regarded as significantly different. The CI plot was also used to reveal if these pairwise differences were significant between individuals. The F test (the var.test function) was performed using R, version 3.1.0 (http://www.r-project.org/), to analyze whether the variation in AIL spermatozoa (million/straw) between batches within bull was different between B and SV semen, i.e., within-bull variation for B and SV.

Moreover, testing for the possible effect of proportion of AIL spermatozoa (million/straw) on outcome of AI measured as 56-day NRR, was analyzed using the general linear model procedure software, using SAS version 9.3 (SAS 2002–2010) for Microsoft Windows (SAS Institute Inc.) by the following model:

$$Y_{ijklmn} = \mu + p_i + t_j + (b_1 \times X_{ijk}) + e_{ijk}$$

where:
Y_{ijk} = observation per heifer/cow;
μ = overall mean;
i = effect of month of AI, k = 1 to 12;
t_i = effect of parity, l = 0 to 4;
b_1 = partial regression of AIL (million/straw) on NRR.
e_{ijk} = random error.

Outcomes with probability values less than 0.05 were considered statistically significant for all analyses performed.

3. Results

3.1. Field fertility

The motility analyses from the 170 batches included in the study showed no difference in percentage postfreeze motility for B and SV semen, being 55 (4), mean (standard deviation) for both. Nonreturn rates (56 days) were obtained for AIs performed during 12 months from the start of the trial, giving data on a total of 14,125 first inseminations from 166 batches, 7081 with B and 7044 with SV semen, Table 1. There was a significant influence of both month of AI, bull, and parity of the females on 56-day NRR. Most AIs were performed during the months September through December, representing 87.4% and 87.8% of total AIs for B and SV, respectively. The number of AIs with B and SV was well balanced within month, maximum difference being 0.3% of total number of AIs per semen treatment. Artificial inseminations performed in the months of June, August, and September resulted in significant superior NRR in comparison with the other months, representing 15.5% and 15.0% of all AIs for B and SV, respectively. All 16 bulls had a well-balanced number of AIs within semen treatment. Artificial inseminations from three bulls resulted in significantly higher NRR compared to the remaining 13 bulls. Parity of the females represented highly significant ($P < 0.01$) influence on the NRR (Table 1). There was, however, no significant difference in 56-day NRR for the two semen categories, neither overall or when statistical analyses were performed within month of AI, bull or parity. Overall NRRs presented as least-square means were 72.5% and 72.7% for B and SV, respectively.

When the variable bull in the least squares analysis was replaced by batch (i = 1–166), the possible influence of variation between batches on NRR was estimated. There was no significant influence of batch variation, neither when statistical analyses were performed overall nor within semen processing methods.

3.2. Plasma membrane and acrosome integrity assessed by flow cytometry

Semen samples from all 170 batches assessed for plasma membrane and acrosome integrity resulted in mean AID, ARD, AIL, and ARL spermatozoa for the B semen batches being 25.0% (0.06), 23.7% (0.07), 49.3% (0.11), and 1.5% (0.01), respectively. For the SV samples, corresponding values were 27.1% (0.07), 33.9% (0.08), 36.0% (0.12), and 2.4% (0.01). Mean values with 95% CI are presented in Figure 1.

These results show a significant effect ($P < 0.0001$) of processing method, with the SV technology resulting in a lesser proportion of percent AIL spermatozoa than for

Table 1
Least-square means for 56-day nonreturn rate (NRR) for artificial inseminations (AIs) with Biladyl- (B) and SpermVital- (SV) processed semen within parity (parity 0: heifers; parity 4: ≥4 lactations) with corresponding number of AIs.

Parity	B		SV	
	NRR (%)	No. of AIs	NRR (%)	No. of AIs
0	77.4	2516	77.3	2436
1	69.2	1928	70.2	2005
2	71.2	1251	71.7	1228
3	72.0	669	71.5	681
4	73.4	717	71.9	694
Total	72.5	7081	72.7	7044

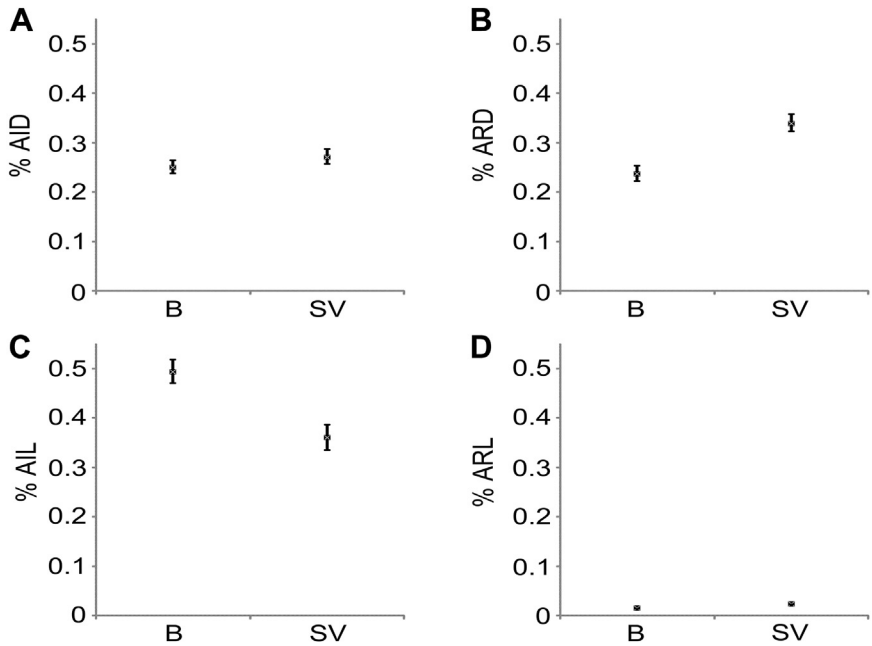

Fig. 1. (A–D) 95% Confidence interval plot with upper and lower confidence limits and mean values measured as (A) percentages of acrosome-intact dead spermatozoa (% AID), (B) acrosome-reacted dead spermatozoa (% ARD), (C) acrosome-intact live spermatozoa (% AIL), and (D) acrosome-reacted live spermatozoa (% ARL) for 170 batches of Biladyl (B) and SpermVital (SV) semen.

semen processed by the ordinary method. The processing method explained 19.5% of the total variation in percentage of AIL spermatozoa. Moreover, there was a significant effect ($P < 0.0001$) of bull, explaining 17.0% of the total variation in measured percent AIL spermatozoa. Neither differences between straws within batch nor replicate within straw affected percentages of AIL spermatozoa significantly.

The control experiment, performed to evaluate the possible influence of the difference in sperm concentrations on the outcome of sperm quality analyses, showed that there were no significant differences in percentages of AIL spermatozoa between semen samples processed with 12 million sperm/straw or with 25 million sperm/straw within processing method. However, percentages of AIL spermatozoa were significantly lower in SV semen, 28.0% (5.3) and 31.3% (5.9), than those in B semen, 46.4% (4.4) and 47.9% (3.6), in doses with 12 and 25 million sperm/straw, respectively.

3.3. Variation in percentages of AIL spermatozoa between B and SV semen in association to AI outcome

When comparing the AIL data transformed to millions of spermatozoa per straw, the CI for paired difference in AIL spermatozoa (million/straw) between SV and B ($\Delta AIL_{SV\text{-}B}$) semen showed a significant difference between the processing methods within bull for nine of the 16 bulls (Fig. 2). Seven of the paired differences are crossing the reference line at zero (Fig. 2) and therefore considered not significantly different. In addition, the results showed that there was a significant difference in $\Delta AIL_{SV\text{-}B}$ spermatozoa between some of the individuals. As an example, bulls 2 and 5 show a significant difference in $\Delta AIL_{SV\text{-}B}$ (million/straw) spermatozoa, but the $\Delta AIL_{SV\text{-}B}$ (million/straw) spermatozoa for bull 1 is not significantly different from bull 5. The results also showed that there was a variation in width of the CIs, indicating variation in $\Delta AIL_{SV\text{-}B}$ (million/straw) spermatozoa across the individuals. Further, the *F* test showed that the

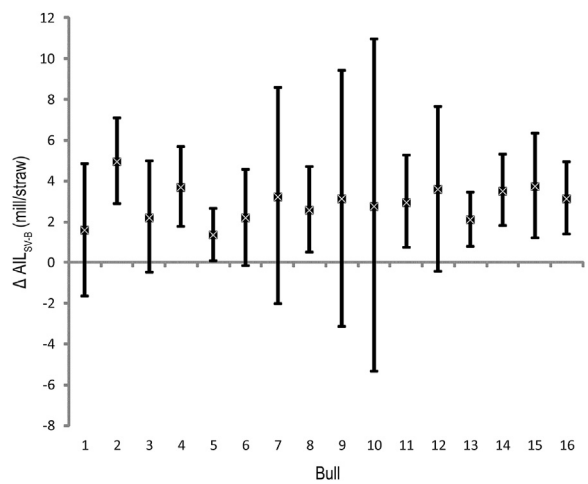

Fig. 2. 95% Confidence interval plot with upper and lower confidence limits and mean values measured as millions of acrosome-intact live (AIL) spermatozoa per straw for each bull (n = 16) on the basis of data from a paired *t* test measured as differences (Δ) in AIL (million/straw) spermatozoa between SpermVital (SV) and Biladyl (B) semen samples (Δ $AIL_{SV\text{-}B}$). The figure shows the reference line at zero used to decide if this pairwise difference was significantly different within individuals.

variance in AIL spermatozoa (million/straw) for SV semen was significant higher than that for B semen, i.e., higher variance between batches within individual and also between bulls processed by SpermVital technology ($F_{82,82} = 5.14$, $P < 0.0001$).

The level of AIL spermatozoa (million/straw) did, however, not influence the 56-day NRR significantly when included as an explanatory variable in the general linear models procedure, neither overall nor within semen processing method.

4. Discussion

The field fertility results measured as 56-day NRR were equal for AIs with standard B and immobilized SV semen in this trial. Because the trial had a blinded design, i.e., semen processing of AI dose was unknown to the technicians and veterinarians, the AIs were performed at conventional timing relative to ovulation on the basis of estrous signs and observations thereof. Recommendation in Norway is to inseminate during the second half of the estrus and the first six hours after termination of estrous signs, theoretically being the period from 9 to 24 hours after the onset of estrus and 6 to 21 hours before ovulation. The SpermVital technology has been developed to make timing of AI less critical, by extending sperm survival at body temperature and to allow a gradual release of spermatozoa over time after AI. The question raised in this investigation was whether enough sperm cells are released and capable of fertilization at conventional timing of AI. Previous publications on encapsulated spermatozoa have conflicting results concerning AI on animals in estrus. In some publications, the authors conclude that encapsulated sperm cells are capable of fertilizing *in vivo* but are not favored by insemination at conventional time [7,19], whereas others report improved fertility at optimum time using encapsulated spermatozoa [8].

The SpermVital technology is using a quite different approach than encapsulation described previously, as sperm cells are embedded in an alginate gel, which will gradually dissolve and release sperm cells over an extended period of time [14]. The number of sperm cells released per hour *in utero* and the dissolving period are not exactly known. Cows have been slaughtered after AI with SV semen showing alginate gel residues being present *in utero* approximately 24 hours after AI (unpublished data). Several *in vitro* studies applying forced dissolution of the gel over different time periods have reported continuous and gradually sperm relase during the dissolving period (unpublished data). This is in contrast to encapsulation where sperm cells are located in a liquid core surrounded by an alginate capsule [7]. On capsule rupturing, all sperm cells are released immediately, and only asynchronous rupturing of capsules will ensure extended sperm release. Another difference is that encapsulated sperm cells are in a liquid environment, thus not physically immobilized, and possibly precapacitated because of the encapsulation procedure [8]. In theory, the gradual dissolution of SV semen should ensure availability of a limited population of spermatozoa ready to fertilize also when AI is performed at optimum timing. The fertility data from this study confirms that SV semen shows equal performance to conventional semen when inseminated at estrus.

In this study, heifers showed significantly higher fertility rates than cows. Moreover, the month at which the AIs were performed significantly influenced NRR. These results are in compliance with fertility recording in Norway [20–22]. Fertility in NRF is high compared to other cattle breeds [23] mainly because of having emphasized fertility performance in the NRF breeding program for decades [24,25]. Obtaining equal NRR with immobilized spermatozoa in comparison to standard processed semen is thus a good demonstration of the technology's performance, as improvement of already high fertility rates is difficult to report.

The fact that SV semen contained about twice the number of sperm cells as standard semen might have influenced the results, as it is known that fertility potential of a semen sample is affected by the number of spermatozoa per AI dose [26,27]. An increasing number of spermatozoa per AI dose will improve fertility up to a certain threshold, when the maximum fertility level of the bull is reached and where it is no longer possible to compensate for the differences in fertility between the males by increasing the number of spermatozoa per AI dose [27,28]. Previously, it has been shown with conventional semen that AIs with 12 million sperm cells/dose perform similarly to AIs with 18 million/dose [29]. Consequently, the AI dose of 12 million sperm/straw used for B semen in the present study should be regarded as above the threshold of maximum fertility [26,27], at least for most bulls. Also, 12 millions were chosen because this was the standard sperm number per dose used for NRF young bulls used in this study (Geno SA, Hamar, Norway). However, the SV semen was produced with a higher sperm concentration because of the theory that this semen is dissolved gradually after insemination, i.e., to ensure enough available spermatozoa throughout the dissolving period. Dissolution of the alginate gel and thereby sperm release is believed to last for at least 24 hours. Additionally, the higher number of spermatozoa per AI dose was also a compensation for the reduced postthaw sperm survival caused by the immobilization procedure. In contrast to experiments on encapsulation finding no or minimal sperm injuries due to the technology [5,6,30], immobilization in combination with cryopreservation caused increased sperm death compared to conventional semen processing injuries as reported by *in vitro* analyses in the present study.

The final sperm concentration after processing of semen might have had an effect on the postthaw sperm quality. In the present study, SV semen had a sperm concentration of about the twice that of B semen. The control experiment, however, confirmed that the difference in sperm concentration between B and SV semen did not influence the postthaw proportion of AIL spermatozoa. Thus, the observed differences in sperm quality between B and SV semen should therefore solely be influenced by the processing method.

The ANOVA test showed that there was a significant effect of processing method, as the first-generation SV technology used for immobilization and cryoconservation in this study resulted in a lower proportion of percent AIL spermatozoa than semen processed by the standard method. In

accordance with other studies, bull significantly affected the proportion of AIL spermatozoa (%) postthaw [31].

The CI for paired difference in AIL spermatozoa (million/straw) between B and SV semen showed that there was a variation in width of the confidence interval, indicating that different bulls show a different tolerance to the two semen processing methods. In fact, the result indicates that semen from some bulls tolerates immobilization and cryopreservation better than other ones, a fact that must be taken into account in further improvement of the technology.

No association was found between the *in vitro* sperm quality results measured as AIL spermatozoa (million/straw) and the fertility results. This result is in accordance with earlier studies showing that *in vitro* assessment of viability and acrosome integrity had no correlation to field fertility [17,31,32], whereas others have shown an association [16,33,34]. The reason for these conflicting results could be due to the relatively high number of sperm cells in the semen samples of both B and SV used for AI, i.e., there are still enough sperm cells capable of fertilizing the egg even in the samples with the poorest quality in this study. It is also possible that assessment and characterization of sperm subpopulations will elucidate association of sperm quality with fertility. As an example, Birck et al. [34] found that the percentage of acrosome reaction inducibility in the live sperm population appeared to be a better sperm quality parameter for prediction of bull fertility. It is also possible that including a combination of several sperm quality parameters might be a good predictive model for field fertility, as others have claimed earlier [33].

4.1. Conclusions

The present study shows that semen processed by SpermVital immobilization technology performs equally to standard processed semen when AI is performed at conventional time, although the immobilization procedure caused increased sperm damage evaluated *in vitro*. The fact that the AI was performed at conventional timing is regarded as suboptimal for SpermVital-processed semen compared to Biladyl-processed semen on the basis of the theory of gradually dissolving of the gel and thereby gradually release of the spermatozoa in a SpermVital dose. SpermVital AS will continue to develop the immobilization technology to improve sperm quality and production efficiency.

Acknowledgments

The field study was conducted as part of a project granted by the Norwegian Research Council (project number 207799). The flow cytometric *in vitro* studies were funded by a PhD scholarship at Hedmark University College, financed by the Norwegian Government, Ministry of Education and Research and are part of a project collaboration between Hedmark University College, Geno SA and Norwegian University of Life Sciences. The authors are grateful for advice from Arne Linløkken (Hedmark University College) regarding statistical analyses and would like to thank Geno SA for producing, distributing, and providing samples for this study.

References

[1] Hafez ESE. Reproduction in farm animals. Sixth edition. Philadelphia: Lea & Febriger; 1993.
[2] Polge C, Smith AU, Parkes AS. Revival of spermatozoa after vitrification and dehydration at low temperatures. Nature 1949;164:666.
[3] Smidsrod O, Skjak-Braek G. Alginate as immobilization matrix for cells. Trends Biotechnol 1990;8:71–8.
[4] Strand BL, Morch YA, Skjak-Braek G. Alginate as immobilization matrix for cells. Minerva Biotecnol 2000;12:223–33.
[5] Nebel RL, Bame JH, Saacke RG, Lim F. Microencapsulation of bovine spermatozoa. J Anim Sci 1985;60:1631–9.
[6] Munkittrick TW, Nebel RL, Saacke RG. Accessory sperm numbers for cattle inseminated with protamine sulfate microcapsules. J Dairy Sci 1992;75:725–31.
[7] Nebel RL, Vishwanath R, McMillan WH, Saacke RG. Microencapsulation of bovine spermatozoa for use in artificial insemination: a review. Reprod Fertil Dev 1993;5:701–12.
[8] Vishwanath R, Nebel RL, McMillan WH, Pitt CJ, Macmillan KL. Selected times of insemination with microencapsulated bovine spermatozoa affect pregnancy rates of synchronized heifers. Theriogenology 1997;48:369–76.
[9] Nebel RL, Vishwanath R, McMillan WH, Pitt CJ. Microencapsulation of bovine spermatozoa: effect of capsule membrane thickness on spermatozoal viability and fertility. Anim Reprod Sci 1996;44:79–89.
[10] Maxwell WMC, Nebel RL, Lewis GS. Survival and fertility of microencapsulated ram spermatozoa stored at 5°C. Reprod Domest Anim 1996;31:665–73.
[11] Torre ML, Faustini M, Norberti R, Stacchezzini S, Maggi L, Maffeo G, et al. Boar semen controlled delivery system: storage and in vitro spermatozoa release. J Control Release 2002;85:83–9.
[12] Kemmer C, Fluri DA, Witschi U, Passeraub A, Gutzwiller A, Fussenegger M. A designer network coordinating bovine artificial insemination by ovulation-triggered release of implanted sperms. J Control Release 2011;150:23–9.
[13] Weber W, Rimann M, Schafroth T, Witschi U, Fussenegger M. Design of high-throughput-compatible protocols for microencapsulation, cryopreservation and release of bovine spermatozoa. J Biotechnol 2006;123:155–63.
[14] Kommisrud E, Hofmo PO, Klinkenberg G. Preservation and controlled delivery/release of spermatozoa. European Patent No. EP2048943B1/US patent No. 8,178,130 B2. WIPO, World Intellectual Property Organization, Patentscope; 2008.
[15] Watson PF. The potential impact of sperm encapsulation technology on the importance of timing of artificial insemination: a perspective in the light of published work. Reprod Fertil Dev 1993;5:691–9.
[16] Correa JR, Pace MM, Zavos PM. Relationships among frozen-thawed sperm characteristics assessed via the routine semen analysis, sperm functional tests and fertility of bulls in an artificial insemination program. Theriogenology 1997;48:721–31.
[17] Nagy S, Jansen J, Topper EK, Gadella BM. A triple-stain flow cytometric method to assess plasma- and acrosome-membrane integrity of cryopreserved bovine sperm immediately after thawing in presence of egg-yolk particles. Biol Reprod 2003;68:1828–35.
[18] Standerholen FB, Myromslien FD, Kommisrud E, Ropstad E, Waterhouse KE. Comparison of electronic volume and forward scatter principles of cell selection using flow cytometry for the evaluation of acrosome and plasma membrane integrity of bull spermatozoa. Cytometry A 2014;85:719–28.
[19] Nebel RL, Saacke RG. Technology and applications for encapsulated spermatozoa. Biotechnol Adv 1994;12:41–8.
[20] Garmo RT, Refsdal AO, Karlberg K, Ropstad E, Waldmann A, Beckers JF, et al. Pregnancy incidence in Norwegian Red cows using nonreturn to estrus, rectal palpation, pregnancy-associated glycoproteins, and progesterone. J Dairy Sci 2008;91:3025–33.
[21] Geno. Annual report 2011 Available at: http://www.geno.no/Start/Geno-Avler-for-bedre-liv/Om-Geno-SA/Arsberetning-regnskap-og-statistikk/. Accessed October 20, 2013.
[22] Refsdal AO. Reproductive performance of Norwegian cattle from 1985 to 2005: trends and seasonality. Acta Vet Scand 2007;49:5.
[23] Ferris CP, Patterson DC, Gordon FJ, Watson S, Kilpatrick DJ. Calving traits, milk production, body condition, fertility, and survival of Holstein-Friesian and Norwegian Red dairy cattle on commercial dairy farms over 5 lactations. J Dairy Sci 2014;97:5206–18.

[24] Andersen-Ranberg IM, Klemetsdal G, Heringstad B, Steine T. Heritabilities, genetic correlations, and genetic change for female fertility and protein yield in Norwegian dairy cattle. J Dairy Sci 2005;88:348–55.
[25] Heringstad B, Larsgard AG. Correlated selection responses for female fertility after selection for high protein yield or low mastitis frequency in Norwegian Red cows. J Dairy Sci 2010;93:5970–6.
[26] Den Daas JH, De Jong G, Lansbergen LM, Van Wagtendonk-De Leeuw AM. The relationship between the number of spermatozoa inseminated and the reproductive efficiency of individual dairy bulls. J Dairy Sci 1998;81:1714–23.
[27] Amann RP, Hammerstedt RH. Detection of differences in fertility. J Androl 2002;23:317–25.
[28] Saacke RG, Dalton JC, Nadir S, Nebel RL, Bame JH. Relationship of seminal traits and insemination time to fertilization rate and embryo quality. Anim Reprod Sci 2000;60-61:663–77.
[29] Kommisrud E, Steine T, Graffer T. Comparison of fertility rates following insemination with different numbers of spermatozoa per insemination dose of frozen bovine semen. Reprod Domest Anim 1996;31:359–62.
[30] Esbenshade KL, Nebel RL. Encapsulation of porcine spermatozoa in poly-lysine microspheres. Theriogenology 1990;33:499–508.
[31] Waterhouse KE, Haugan T, Kommisrud E, Tverdal A, Flatberg G, Farstad W, et al. Sperm DNA damage is related to field fertility of semen from young Norwegian Red bulls. Reprod Fertil Dev 2006;18:781–8.
[32] Nagy S, Hallap T, Johannisson A, Rodriguez-Martinez H. Changes in plasma membrane and acrosome integrity of frozen-thawed bovine spermatozoa during a 4 h incubation as measured by multicolor flow cytometry. Anim Reprod Sci 2004;80:225–35.
[33] Gillan L, Kroetsch T, Maxwell WM, Evans G. Assessment of in vitro sperm characteristics in relation to fertility in dairy bulls. Anim Reprod Sci 2008;103:201–14.
[34] Birck A, Christensen P, Labouriau R, Pedersen J, Borchersen S. In vitro induction of the acrosome reaction in bull sperm and the relationship to field fertility using low-dose inseminations. Theriogenology 2010;73:1180–91.