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Semen parameters of cloned bulls

J.F. SMITH, R.M. MCDONALD, D.N. WELLS and N.R. COX

AgResearch Limited, Ruakura Research Centre, Hamilton, New Zealand.

ABSTRACT

Bulls cloned from the somatic cells of genetically elite sires can make a major contribution to genetic improvement and the rate of dissemination of improved genetics within both the dairy and beef industries. However, little is known about the physiology of clone males and the quality of their semen in particular. This paper reports on the semen characteristics of five cloned Friesian bulls, compared to the two 'Donor' bulls from which three and two of the clones were derived by nuclear transfer, respectively, and an age matched control bull. Three batches of frozen semen from each of these eight bulls were obtained from a commercial AB centre and analysed. Four straws from each batch of semen were thawed, pooled and made up to 1mL with RSD-1 diluent and sub-samples taken to evaluate viability, motility, morphology, migration (using a synthetic mucus) and DNA damage. Viability, motility and migration assays were performed immediately after thawing (0 hours) and repeated after 3 hours of incubation at 38°C. An additional straw from each semen batch was used to determine sperm concentration and proportion of diploid cells. There were significant differences ($P < 0.05$) between individual bulls in all parameters except diploid cells ($0.16 \pm 0.05\%$), sperm DNA damage ($4.41 \pm 1.13\%$) and normal sperm ($93.3 \pm 6.7\%$). The differences in viability (range 74.9 to 54.9% at 0 hours; 49.6 to 22.5% at 3 hours), motility (59.2 to 28.3%; 52.5 to 29.2%) and migration (vanguard distance 68.3 to 42.5 mm; 38.8 to 12.5mm and running distance (>150 sperm) 33.3 to 23.3mm; 20.4 to 6.7mm) were greater at 3 hours than at 0 hours reflecting individual variation in rate of change in parameters over time. However, examination of the variation between clones within a clonal family or line and between lines revealed that this was similar to the overall individual variation, indicating that members of a clonal family are not more uniform. Nonetheless, these results indicate that the samples of semen from the cloned bulls were as good as those of the original donor bulls and fit for commercial use to potentially disseminate elite genotypes.

Keywords: bulls; clones; semen quality.

INTRODUCTION

Cloned bulls produced following somatic cell nuclear transfer (NT) can make a major contribution to genetic improvement and the rate of dissemination of improved genetics within the beef industry via natural mating and in the dairy industry to satisfy unmet international demands for frozen semen from elite bulls. However, such contributions will be dependant on their being able to produce good quality semen that additionally can withstand cryopreservation. Little is known about the physiology of clone males and the quality of their semen in particular although some recent reports on small numbers have indicated that cloned bulls produce semen suitable for normal use (Lessard *et al.*, 2003; Heyman *et al.*, 2004; Shiga *et al.*, 2005; Tecirlioglu *et al.*, 2006). This information is required if cloned bulls are to be used for breeding purposes at some time in the future, after regulatory approval (Rudenko & Matheson, 2007) and market acceptance (Suk *et al.*, 2007).

While there is no substitute for the actual

insemination of large numbers of cows to determine the fertility of cloned bulls, this is both highly expensive and impractical. The use of *in vitro* evaluation of semen samples through the measurement of a range of sperm parameters has commonly been used to provide some indication of the potential fertility of those semen samples and of the bull. Recent analysis of many such reports suggests that no single parameter is a useful indicator and that a range of parameters that measure a variety of sperm functions is desirable (Rodriguez-Martinez, 2003). Thus in this study a total of 14 sperm parameters examining membrane integrity, motility, chromosomal damage and ability to migrate through mucus were undertaken in frozen and thawed semen samples from cloned bulls from two separate clonal families (a set of NT clones derived from the same source of donor cells) compared to the original donor bulls from which they were derived. In addition, a small scale AI trial compared the pregnancy rates with semen from bulls from one of the clonal families with the original donor sire.

MATERIALS AND METHODS

Reagents

Unless otherwise stated chemicals were of analytical grade. The following products were used:- FertiLight™ Sperm Viability Kit purchased from Molecular Probes Inc (Eugene, USA); TruCount™ Tubes (Becton Dickinson, San Jose, CA, USA); Dulbeccos PBS (1X) from Gibco, NZ; BSA from ICPBio, NZ; acridine orange (AO) purchased from Molecular Probes Inc. (Eugene, USA); hyaluronic acid (HA) 10mg/mL purchased from Vetrapham, (BOMAC Laboratories, NZ); rectangular capillary tubes purchased from VitroCom (New Jersey, USA); BEEM Standard Embedding Capsules #RB001 from ProSciTech (Qld, Australia). All other chemicals were purchased from Sigma Chemical Company (St Louis, MO, USA) and BDH Chemicals Ltd (Poole, UK).

Flow cytometer

A Becton Dickinson FACScan with built in air cooled argon-ion laser, (excitation 488 nm) was used in this study. The green Fluorescence (FL1) was collected through a 515-545 nm band pass filter and the red fluorescence (FL3) through a 650nm long pass filter. Data was acquired and analyzed using Cell Quest™ Becton Dickinson Software unless otherwise stated.

Animals

Semen samples from a total of eight bulls were studied. Five cloned Friesian bulls were produced following NT, representing two clonal families, or lines, denoted AESF4 and AESF5 (Obach & Wells, 2003). Three bulls were generated from the AESF4 line and were referred to as Clone 4.1, 4.2 and 4.3 and an additional two bulls were generated from the AESF5 line and termed Clone 5.1 and 5.2. Semen was collected from the cloned bulls when they were around 17 months of age. The clones were compared with the two 'original' bulls from which they were respectively derived (denoted Donor 4 and Donor 5), along with an age matched 'control' bull.

Semen

Semen was collected and cryopreserved according to the commercial practice at Ambreed Ltd. Three batches of frozen semen from each of these eight bulls were obtained and analysed. Four straws from each batch of semen were thawed, pooled and made up to 1 mL with RSD-1 diluent (Upreti *et al.*, 1995) and sub-samples taken to evaluate a range of semen parameters.

Sperm viability

Viability was measured using the LIVE/DEAD Sperm Viability Kit at a concentration of approximately 50×10^6 /mL by flow cytometry (Garner *et al.*, 1994) at 0 hours and after 3 hours of incubation at 38°C.

Visual Motility

Motility was assessed subjectively by light microscopy by one operator after sperm had been diluted in RSD-1 to a concentration of approximately 100×10^6 /mL at 0 hours and 3 hours after incubation at 38°C (Evans & Maxwell, 1987).

Sperm migration

The extent of sperm migration through a synthetic mucus hyaluronic acid (HA) in rectangular capillary tubes (internal dimensions: 0.3 mm x 3 mm x 100 mm) was determined using the method of McPhie (2002). Marked capillary tubes preloaded with warmed HA diluted 3:1(v/v) with Dulbeccos PBS (1X solution, pH 7.3, mOsm280) + 1% (v/v) BSA were placed vertically into 50µl aliquots of semen (100×10^6) in warmed BEEM Capsules and incubated for 30 minutes at 38°C in a dry incubator. The capillary tube was then placed under a light microscope at 100x magnification and the number of sperm within each marked 5mm segment was counted. The following parameters were recorded. Vanguard distance (distance the most forward moving sperm had reached); Peak distance (distance reached by >10 sperm) and Running total distance (distance reached by >150 sperm). This test was performed at 0 hours and again after 3 hours incubation at 38°C.

Sperm concentration and proportion of diploid cells

An additional straw was thawed for this determination by flow cytometry using TruCount™ Tubes as described by Krzyzosiak *et al.* (2002).

Sperm morphology

Sperm were stained with Eosin/Nigrosin on slides pre-warmed to 38°C. One hundred sperm cells were examined using morphology characteristics depicted in 'Salamon's Artificial insemination of Sheep and Goats' (Evans & Maxwell, 1987).

Sperm Chromatin Structure Assay

Sperm chromatin structure was assessed by flow cytometry using the Sperm Chromatin Structure Assay (SCSA; Evenson & Jost, 1994) to measure the susceptibility of the sperm nuclear

DNA to acid induced denaturation *in situ* by the metachromatic fluorescent shift of acridine orange fluorochromes. Data was acquired by Cell Quest™ Becton Dickinson Software. List mode data were then analysed using the software program FCS Express V2 (De Novo Software, Ontario, Canada).

Artificial Insemination (AI)

Frozen and thawed semen from the three AESF4 cloned bulls and from the Donor 4 bull was used to AI cows that were either clones or the daughters of cloned cows. Pregnancy rates, determined by ultrasonography on around day 80 of gestation, were compared with approximately equal numbers of females being inseminated with the cloned or donor semen.

Statistical Analyses

The eight bulls were partitioned into three lines (AESF4, AESF5, control) with four, three, and one bull per line respectively. T tests were used to determine if the clones in a line were different from the donor of that line. Genstat REML (Residual maximum likelihood) was used to fit (mixed) model to estimate the variance components due to line, bull within line, batch within bull and aliquot within batch.

RESULTS

The mean values (±se) for the 14 semen parameters for each of the eight bulls are presented in Table 1. These indicate a relatively high level (64.2%) of viable sperm immediately post-thaw for all bulls. The percent motile sperm values are within the normal expected range. All bulls showed a high percentage of normal sperm (93.3%) with very low numbers of diploid cells (0.16%) and little chromosomal damage (4.41%). Sperm migration values indicated the ability of most samples to penetrate the synthetic mucus, although there was greater between batch within bull and between lines after 3 hours of incubation than at 0 hours. Those parameters recorded at 0 hours and again after 3 hours all indicated a decrease with time of incubation as expected.

The comparison of the clones with their respective donor bulls indicate that for the AESF5 line there was no significant differences in any of the sperm parameters or in the change over time for viability, motility or migration. In contrast, the AESF4 line showed significant differences between the clones and the donor bull for the viability parameters and the migration running

Table 1: Mean semen parameters (± se) for each bull.

	Bull	Clone 4.1	Clone 4.2	Clone 4.3	Donor 4	Clone 5.1	Clone 5.2	Donor 5	Control	Overall Average
Parameter / Line	AESF4	AESF4	AESF4	AESF4	AESF4	AESF5	AESF5	AESF5	Control	
Viability 0h (%)	69.9 (5.2)	74.9 (2.5)	72.2 (2.7)	59.8 (9.1)	54.9 (6.1)	67.6 (6.2)	55.1 (7.10)	59.2 (2.9)	64.2 (5.2)	
Viability 3h (%)	28.7 (7.2)	33.5 (5.7)	27.7 (1.4)	49.6 (7.6)	22.5 (8.3)	34.4 (12.1)	24.6 (1.9)	27.3 (3.2)	31.0 (5.9)	
Motility 0h (%)	42.5 (17.8)	59.2 (4.9)	50.0 (7.1)	44.2 (8.0)	28.3 (11.3)	54.2 (9.2)	44.2 (4.9)	41.7 (6.2)	45.5 (8.7)	
Motility 3h (%)	35.8 (11.6)	52.5 (6.1)	40.8 (5.8)	30.8 (9.7)	29.2 (4.9)	52.1 (8.4)	39.2 (7.4)	38.3 (7.8)	39.8 (7.7)	
Vanguard 0h (mm) ¹	42.5 (12.9)	55.8 (8.6)	57.5 (21.9)	59.2 (22.5)	55.0 (20.5)	65.8 (28.5)	68.3 (24.6)	60.4 (15.6)	58.1 (19.4)	
Vanguard 3h (mm) ¹	17.5 (4.2)	20.0 (4.5)	25.8 (4.9)	12.5 (4.2)	36.7 (20.7)	21.7 (7.5)	15.0 (0.0)	38.8 (20.1)	23.5 (8.3)	
Peak 0h (mm) ²	36.7 (10.8)	45.0 (6.3)	45.0 (22.8)	36.7 (11.3)	37.5 (14.4)	51.7 (22.3)	47.5 (11.3)	43.3 (8.9)	42.9 (13.5)	
Peak 3h (mm) ²	12.5 (4.2)	14.2 (3.8)	15.8 (2.0)	7.5 (4.2)	20.8 (5.8)	15.0 (0.0)	10.0 (0.0)	28.3 (15.4)	15.5 (4.4)	
Running total 0h (mm) ³	25.0 (7.7)	32.5 (4.2)	26.7 (6.1)	23.3 (4.1)	26.7 (8.2)	31.7 (8.8)	33.3 (6.8)	31.7 (6.9)	28.9 (6.6)	
Running total 3h (mm) ³	10.8 (2.0)	11.7 (2.6)	10.8 (2.0)	6.7 (2.6)	14.2 (6.6)	10.0 (0.0)	10.0 (0.0)	20.4 (9.2)	11.8 (3.1)	
Sperm concentration (10 ⁶ sperm /straw)	20.1 (5.7)	19.5 (2.7)	20.0 (2.7)	13.2 (3.9)	18.9 (1.1)	17.9 (3.6)	20.4 (1.6)	26.7 (7.9)	19.6 (3.7)	
Percent diploid cells	0.145 (0.050)	0.186 (0.044)	0.163 (0.025)	0.109 (0.047)	0.203 (0.074)	0.235 (0.095)	0.104 (0.024)	0.159 (0.031)	0.163 (0.049)	
Percent normal sperm 0h	93.3 (0.6)	95.0 (1.0)	94.7 (2.5)	96.5 (3.5)	97.3 (1.2)	95.7 (3.2)	94.0 (4.4)	87.3 (11.9)	93.3 (6.7)	
Percent chromosome damage	5.02 (1.02)	5.23 (0.65)	5.65 (0.94)	4.22 (1.06)	4.06 (0.37)	3.30 (0.15)	3.85 (0.38)	3.26 (0.66)	4.41 (1.13)	

¹Migration distance of furthest 1 sperm

²Migration distance of furthest 10 sperm

³Migration distance of furthest 150 sperm

total at 3 hours (Table 2). In general for all sperm parameters there were greater differences between clones and donor in this line as indicated by the lower P values shown in Table 2.

Table 2: Comparison of cloned bulls with the respective donor bull within each clonal line, for each semen parameter (P values).

Semen Parameter	AESF4	AESF5
Viability 0h (%)	0.049	0.674
Viability 3h (%)	0.032	0.769
Viability change ⁴	0.004	0.186
Motility 0h (%)	0.576	0.917
Motility 3h (%)	0.341	0.953
Motility change ⁴	0.073	0.333
Vanguard 0h (mm) ¹	0.526	0.554
Vanguard 3h (mm) ¹	0.223	0.472
Vanguard change ⁴	0.129	0.504
Peak 0h (mm) ²	0.423	0.851
Peak 3h (mm) ²	0.074	0.362
Peak change ⁴	0.808	0.644
Running total 0h (mm) ³	0.408	0.512
Running total 3h (mm) ³	0.015	0.667
Running total change ⁴	0.951	0.575
Sperm concentration (10 ⁶ sperm /straw)	0.002	0.251
Percent diploid cells	0.146	0.153
Percent normal sperm	0.167	0.333
Percent chromosome damage	0.101	0.832

¹Migration distance of furthest 1 sperm

²Migration distance of furthest 10 sperm

³Migration distance of furthest 150 sperm

⁴Change in parameter over the 3h incubation at 38°C

Table 3 presents the variance components for the different sperm parameters. This indicates the additional variation attributed to the different components: between aliquots within batches (where applicable), between batches within bull by

line, between bulls within line and between the different lines. These data indicate that for the viability, motility and chromosomal damage parameters there was less within batch variation compared to that between batches within bull by line and a further between bull within line contribution. The component of variation due to bulls within lines was greater than that between lines for viability and motility whereas the chromosomal damage indicated less between bull within line variance compared to that between lines. In contrast, the migration parameters at 0 hours showed a large within batch component and little additional contribution from the other components. However, after 3 hours incubation there were significant between batch contributions to the variance as well as a substantive between lines component. The pregnancy rate following AI with semen obtained from the three AESF4 cloned bulls was 13/18 (72%), with an average of 2.2 inseminations per pregnancy. In comparison, the pregnancy rate with semen from the original Donor 4 was 14/17 (82%), achieved with an average of 2.1 inseminations per pregnancy.

DISCUSSION

The sperm parameter measurements recorded indicates that all the cloned bulls studied were capable of producing semen that was of good quality and capable of withstanding cryopreservation.

The post-thaw characteristics were indicative of semen that should produce satisfactory fertility following artificial insemination (Rodriguez-Martinez, 2003). Indeed, following a small number of inseminations of frozen-thawed semen into

Table 3: Variance components for the different semen parameters. The total variance (mean squared) and the relative percentage contribution of the different variance components is presented.

Semen Parameter	Line	Line.Bull	Line.Bull.Batch	Within batch	Total Variance
Viability 0h	23.62%	31.89%	43.84%	0.65%	96.27
Viability 3h	2.26%	44.13%	49.72%	3.89%	115.31
Motility 0h	0.00%	34.89%	46.88%	18.23%	160.78
Motility 3h	0.00%	39.51%	35.76%	24.73%	122.31
Vanguard 0h ¹	2.04%	0.00%	0.00%	97.96%	391.20
Vanguard 3h ¹	17.60%	11.28%	61.24%	9.89%	219.59
Peak 0h ²	0.00%	0.00%	0.87%	99.13%	194.90
Peak 3h ²	46.67%	2.87%	38.97%	11.49%	113.20
Running total 0h ³	3.72%	9.38%	7.44%	79.47%	54.06
Running total 3h ³	51.39%	0.00%	37.4%	11.16%	49.44
Sperm concentration †	37.53%	0.22%	62.25%		36.53
Diploid cells†	0.00%	22.7%	77.24%		0.00
Normal sperm	25.59%	0.00%	2.49%	71.92%	48.50
Chromosome damage	50.38%	2.42%	43.19%	4.01%	1.37

¹Migration distance of furthest 1 sperm

²Migration distance of furthest 10 sperm

³Migration distance of furthest 150 sperm

†No within batch variance component as only a single aliquot measurement made for each batch for this parameter

females that were either clones or daughters of cloned cows showed that the pregnancy rate was similar to that achieved with semen from the original donor into comparable females. This data is in agreement with that of others who have shown that sperm characteristics and rates of *in vitro* embryo production, pregnancy and calving were not different between cloned bulls and their respective donors or controls (Heyman *et al.*, 2004; Shiga *et al.*, 2005; Tecirlioglu *et al.*, 2006).

The analyses of the data showed that for some sperm parameters the different clonal lines differed in their variation and in particular in the difference between the clones and the donor bull. This difference between clonal lines is also seen in the overall efficiency of cloning and in the abnormalities and problems encountered in the cloning process (Heyman *et al.*, 2002; Powell *et al.*, 2004). With the exception of the viability and motility parameters, the analyses of the variance components indicate that the variation between clones within a line is generally less than that between lines. However any such variation is generally not significantly greater than that between batches of semen from the individual bulls within clonal lines. Similar variation between batches of frozen semen have been reported elsewhere and also in other species (Salamon & Maxwell, 1995a; 1995b). A major contributor to this will be the differences in the seminal plasma contribution to the different ejaculates collected (Smith *et al.*, 1999) and the effects that different components of the seminal plasma have on the freezability of sperm (Maxwell *et al.*, 1999).

The best use of clones in agriculture is for breeding to transmit superior genotypes, so long as they remain faithful genomic copies of the original donor animal and that trans-generational (epi)genetic inheritance does not lead to deleterious long-term breeding consequences. Whilst normal telomere lengths have been observed in the spermatozoa of somatic cell cloned bulls and subsequent progeny (Miyashita *et al.*, 2003), this may not always be the case. It has been reported that the progeny of cloned goats did indeed inherit shorter telomeres, similar to their cloned parents (Betts *et al.*, 2005) although this may be dependent on the type of donor cell used for NT (Miyashita *et al.*, 2002). Breeding from cloned sires also avoids confounding issues with the transmission of mitochondrial DNA, which is only maternally inherited (Sutovsky *et al.*, 2000). Importantly, initial results indicate that the clone-associated phenotypes are not transmitted to offspring following sexual reproduction (Shimozawa *et al.*, 2002; Wells, 2003; Martin *et al.*, 2004), implying they are epigenetic in nature.

Thus any epigenetic errors in the surviving clones appear to be reset or corrected during gametogenesis, although further molecular studies are required to substantiate this. Overall, these observations provide some confidence for the main potential application of cloning technology in agriculture; namely, the generation of cloned sires for breeding. Additionally, at a practical level, it remains to be demonstrated that the daughters of cloned dairy sires, for instance, have a similar phenotypic performance to contemporary progeny of the original donor bull.

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