Theriogenology 114 (2018) 40–45

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

In vitro sperm quality and DNA integrity of SexedULTRA™ sex-sorted sperm compared to non-sorted bovine sperm

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ARTICLE INFO

Article history:
Received 3 January 2018
Received in revised form
16 March 2018
Accepted 16 March 2018
Available online 21 March 2018

Keywords:
SexedUltra™
Conventional
Sperm quality
DNA fragmentation

ABSTRACT

SexedULTRA™ is an improved method of sex-sorting sperm creating a less damaging environment to retain sperm integrity through the sorting process. The aim of this study was to evaluate the *in vitro* characteristics of fresh and frozen bovine sperm using the SexedULTRA™ method, and compare it to conventional (non-sorted) sperm. For both methods, percent total sperm motility was estimated visually and also classified into total and progressively motile using a computer assisted sperm analyzer (CASA). Percent sperm with intact plasma membranes (VIA) and acrosomes (PIA) were assessed using flow cytometry and sperm DNA fragmentation index (DFI) was estimated using the Bull sperm Halomax® Kit. Two contemporaneous ejaculates from 10 bulls were processed and cryopreserved using one of the two procedures (SexedULTRA™ and conventional). Sperm motility, VIA and PIA were assessed post-thaw (0 h) and post-incubation (3 h at 37 °C, 8 h and 24 h at 18 °C). DFI was analyzed post-thaw (0 h) and after 6, 24, 48 and 72 h of incubation at 37 °C. In a second experiment, ejaculates from 7 bulls were split sampled into the two types of processing (SexedULTRA™ and conventional) and diluted using a fresh semen extender developed for sex-sorted bovine sperm. Sperm quality was assessed after dilution (0 h) and after incubation for 12, 24, 48, 72 h at 18°, and the same time points of incubation at 37 °C for DFI. Frozen-thawed SexedULTRA™ sperm was significantly ($P < 0.05$) better than conventional semen after a 3 h incubation at 37 °C for PIA, and after a 24 h incubation at 18 °C for percent visual motility and PIA. DFI was significantly lower for SexedULTRA™ compared to conventional at all time points of incubation (37 °C). Fresh SexedULTRA™ sperm showed improved quality compared to conventional at all time points of incubation at 18 °C for percent visual and total motile sperm, VIA, PIA, and DFI. Significant differences were also found in progressive motile sperm immediately after dilution (0 h), but not at any time point after incubation. The results show that the SexedULTRA™ process maintains the quality of sex-sorted sperm and, in many cases, has better *in vitro* longevity than conventional semen.

1. Introduction

Since the first publications showing that flow cytometry was a feasible and reliable method to separate X and Y chromosome bearing sperm, the process has been subject to continual refinement and is now a commercial product available worldwide [1–4]. Sex-sorted sperm was first introduced commercially by XY Inc. and Cogent in late 2000 in the United Kingdom, using the XY method [5]. Bovine sperm sorting laboratories are now operating in more than 25 locations, in 15 countries, with an estimated annual production of 10 million straws [6]. Most reputable cattle artificial breeding companies offer a sex-sorted sperm option as part of their portfolio and the use of this product is increasing. Earlier, the limitations to a more widespread use of sex-sorted sperm in cattle had been the slow sorting process, which reduced production efficiency and increased costs. The introduction of application specific Genesis™ Digital sperm sorting systems and the use of multi-headed sperm sorters have now reduced this limitation considerably. Increased sorter speeds and associated technology allow operators to produce over 300 sex-sorted straws per sorter head per day [7,8]. Also, it is common to see many Sexing Technologies and associated licensed production laboratories operating more than 10 sorter heads, with some laboratories operating over 70 sorter heads in one location (Jared Templeton, Global Production Manager at Sexing Technologies, personal communication).

The other limitation to sex-sorted sperm is the reduced fertility

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https://doi.org/10.1016/j.theriogenology.2018.03.025
0093-691X/

compared with conventional semen [4,5,9]. Successful sperm sorting must consider the susceptibility of gametes to each of the many stages of the sorting process, including high dilutions, staining at 34–36 °C, laser exposure, sorting in contact with a biocompatible sheath fluid, holding sperm after sorting, centrifugation followed by cryopreservation at low cell concentrations. Recognizing that an improvement in the quality of sex-sorted sperm would allow wider commercial application, a concerted effort in the last few years to understand the biochemistry of extenders used for sperm sex-sorting and the processing methodology, has resulted in substantial changes in media composition and alterations to the various stages of the process. This new method branded SexedULTRA™ was designed to provide a supportive environment that accommodates changes in pH, temperature and tonicity, and retains sperm integrity through the entire process.

The objective of these experiments was to evaluate the effects of the SexedULTRA™ sex-sorting method on *in vitro* sperm quality. We hypothesized that SexedULTRA™ would improve *in vitro* sperm quality resulting in equal or better longevity to that of conventional semen.

2. Materials and methods

2.1. Semen samples

All the animals used in this research were treated in accordance with the Federation of Animal Science Societies (2010) guide for the use of farm animals in research and teaching. Ejaculates from a total of 17 bulls owned by Sexing Technologies were collected via artificial vagina in Navasota (TX, USA) and Fond du Lac (WI, USA). Only ejaculates with a sperm motility ≥65%, and abnormal head ≤15% and tail morphology ≤15%, were included in the analysis.

2.2. Conventional and SexedUltra™ procedures

Conventional semen was processed using standard industry methods. For conventional cryopreserved semen, ejaculates were diluted with a Tris-citrate egg yolk medium at 19 °C, equilibrated to 4 °C for a minimum of 90 min, and then re-diluted to a final concentration of 90 million cells per mL with a Tris-citrate glycerol egg yolk medium. For conventional fresh semen, fresh extender (FSRD4+, Sexing Technologies, TX, USA) was used to adjust sperm concentration to 45 million cells per mL.

Sex-sorted sperm was processed using the basic method of semen dilution and staining as described in Seidel and Garner, 2002 [5] with modifications that are collectively termed SexedULTRA™. This method is a revision of the media and conditions under which semen is processed and sorted, including the use of Genesis™ Digital sperm sorting systems designed in collaboration with Cytonome ST LLC. The media formulations in the SexedULTRA™ method are trade secret and proprietary and protected as intellectual property of Inguran LLC through patent US 9,781,919. SexedULTRA™ sperm was sorted at approximately 90% X chromosome purity. Final concentration of sex-sorted sperm before cryopreservation was 18 million cells per mL. Fresh sex-sorted sperm was diluted with fresh extender (FSRD4+, Sexing Technologies, TX, USA) to 10 million cells per mL.

2.3. Sperm quality determination

Sperm concentration was determined using the SP1-Cassette, Reagent S100, and NucleoCounter SP-100 system (ChemoMetec A/S, Denmark). Visual motility was estimated at 37 °C on 100 sperm cells under bright field microscopy with a Nikon Eclipse 80i microscope (Melville, NY, USA). Motility on a minimum of 500 cells at 37 °C was classified into total and progressively motile using a computer assisted sperm motility analyzer (CASA-IVOS II system, Hamilton Thorne, MA, USA). Percent sperm with intact plasma membranes (VIA) and acrosomes (PIA) were assessed after staining with Hoechst 33342 (Sexing Technologies, TX, USA. Final concentration: 10.0 μg/mL), Propidium Iodide (PI; Life technologies, IL, USA. Final concentration: 2.0 μg/mL) and *Arachis hypogaea* conjugated with fluorescein isothiocyanate (FITC-PNA; Thomas Scientific, NJ, USA. Final concentration: 4.0 μg/mL). Sperm were then incubated at 34 °C for 15 min. A minimum of 10,000 events were analyzed using a modified MoFlo SX sperm sorter with Summit v4.0 software (Beckman Coulter, Miami FL). The sorter was fitted with a Vanguard HMD 350mW/355 nm laser (Spectra Physics, Santa Clara CA) that excited Hoechst 33342 to identify Forward and Side Angle Fluorescence This laser was used to improve accuracy of the analysis, allowing to gate on sperm cells and exclude debris or other material. The sorter was also modified with a Coherent Sapphire OPSL laser (Coherent Inc, San Jose, CA) operating at 488 nm to excite PI emitting at 620 nm and FITC-PNA emitting at 530 nm. The emission from the Coherent laser was split using a DCLP 555 dichroic mirror to divide the light in two separate photomultiplier tubes, one detector path utilized a 620/60 (PI) and the other path used a 530/40 (FITC-PNA) bandpass filter. VIA and PIA were calculated as the percentage of PI and FITC-PNA negative sperm populations, respectively. DNA fragmentation index (DFI) was assessed on 300 sperm cells using the Bull sperm Halomax® commercial Kit (Halotech DNA, Madrid, Spain).

2.4. Experiment 1 – frozen semen

Ejaculates from 10 Holstein bulls were processed at the Sexing Technologies production laboratory in Fond du Lac (WI, USA) and sent for quality analysis to the Research and Development Laboratory in Navasota (TX, USA). Two contemporaneous ejaculates were processed per bull for one of the two procedures (SexedULTRA™ and conventional). 20 million conventional or 4 million SexedULTRA™ sperm cells were cryopreserved per 0.25-mL straw using the automated freezing device IMV Digitcool (IMV, France), and stored under liquid nitrogen. For quality analysis, one conventional and one sex-sorted straw were thawed at 38 °C for 45 s. Contents of each straw were split in two aliquots and placed into pre-labeled 1.5-mL Eppendorf microcentrifuge tubes (Eppendorf North America, NY, USA) at 37 °C and at 18 °C. Post-thaw (0 h) and post-incubation (3 h at 37 °C, 8 h and 24 h at 18 °C) percent visual and CASA sperm motility, VIA and PIA were assessed. DFI was also assessed post-thaw (0 h) and post-incubation (6, 24, 48 and 72 h, at 37 °C) for both semen processing procedures.

2.5. Experiment 2 – fresh semen

Ejaculates from 7 bulls (4 Holstein, 2 Jersey, 1 Brown Swiss) were processed and analyzed at the Sexing Technologies R&D laboratory in Navasota Texas. One ejaculate per bull was split sampled into the two types of processing (SexedULTRA™ and conventional). Fresh extended samples were split in two aliquots and placed into pre-labeled 1.5-mL Eppendorf microcentrifuge tubes at 37 °C and at 18 °C. Percent visual, total and progressively motile sperm, and VIA and PIA were assessed after dilution in fresh extender (0 h) and after incubation at 18 °C (12, 24, 48, 72 h). DFI was analyzed at the same time points but incubated at 37 °C.

2.6. Statistical analysis

The *in vitro* sperm quality data were analyzed by analysis of variance with the fixed effect of treatment and random effect of bull

(JMP 10.0.0; SAS, 2012). The analysis of variance was used in conjunction with a Tukey contrast to analyze the treatment effects across the various time points of incubation. Treatment by time interactions were analyzed to determine collinearity. For all measures, Least-squares means and the standard error of the contrast are reported. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Experiment 1 – frozen semen

The comparative estimates of sperm percent visual motile, total and progressive motility, VIA and PIA between SexedULTRA™ and conventional post-thaw at 0 and 3 h post-incubation at 37 °C are shown in Table 1A. The 8 and 24 h post-incubation at 18 °C results are shown in Table 1B. No significant differences ($P > 0.05$) were found between frozen-thawed SexedULTRA™ and conventional sperm for the post-thaw quality parameters analyzed, with exception to PIA after a 3 h incubation at 37 °C and a 24 h incubation at 18 °C, and percent visual motility after 24 h at 18 °C.

The comparative estimates for sperm DFI post-thaw and 6, 24, 48 and 72 h post-incubation at 37 °C are shown in Fig. 1. Significant differences ($P < 0.05$) were found in DFI between SexedULTRA™ and conventional sperm at 0 h, and post incubation (37 °C) at 6 h, 24 h, 48 h and 72 h.

In all cases, a significant bull effect was observed ($P < 0.05$). A strong time by treatment effect ($P < 0.05$) was found in PIA and DFI parameters during incubation of conventional sperm. This interaction was not present ($P > 0.05$) in SexedULTRA™ sperm, or in percent visual motile, total motile or VIA results for any of the treatments.

3.2. Experiment 2 – fresh semen

The comparative estimates of percent visual, total and progressive motility, VIA and PIA between SexedULTRA™ and conventional post fresh dilution and after 12, 24, 48 and 72 h post-incubation at 18 °C are shown in Table 2. Significant differences ($P < 0.05$) were found between fresh SexedULTRA™ and conventional sperm for all of the parameters analyzed, with exception to progressive motile at 12, 24 and 72 h post-incubation at 18°.

The comparative estimates for sperm DFI at 0, 12, 24, 48 and 72 h post-incubation at 18 °C are shown in Fig. 2. Significant differences were found in DFI between SexedULTRA™ and conventional fresh sperm at all time points of incubation.

In all cases, a significant bull effect was observed ($P < 0.05$). A strong time by treatment effect ($P < 0.05$) was seen in PIA and DFI parameters during incubation of conventional sperm. This interaction was not present ($P > 0.05$) in SexedULTRA™ sperm, or in percent visual motile, total motile or VIA results for any of the treatments.

Table 1A
Comparison of percent visual, total and progressive motility, intact plasma membranes (VIA) and acrosomes (PIA) of frozen-thawed SexedULTRA™ sex-sorted and conventional sperm. Data shown includes LS Means, and Tukey Contrast SE and P-values at 0 and 3 h after incubation at 37 °C. Differences were considered significant at $P < 0.05$ (Bold and underlined values), n = 10.

Value	Time	LS Means		Tukey Contrast		
		Conventional	SexedULTRA™	Change	SE	P
Visual Motile	00 h	61.0	63.8	2.8	2.4	0.250
	03 h	50.1	51.0	0.9	2.4	0.709
Total Motile	00 h	60.6	63.8	3.2	2.2	0.157
	03 h	49.6	50.0	0.4	2.2	0.862
Prog. Motile	00 h	49.8	53.0	3.3	2.5	0.198
	03 h	28.5	29.4	1.0	2.5	0.698
VIA	00 h	55.6	56.7	1.1	1.6	0.502
	03 h	40.7	43.4	2.6	1.6	0.121
PIA	00 h	72.6	76.0	3.3	2.1	0.126
	03 h	55.6	62.3	6.7	2.1	**0.004**

Table 1B
Comparison of percent visual, total and progressive motility, intact plasma membranes (VIA) and acrosomes (PIA) of frozen-thawed SexedULTRA™ sex-sorted and conventional sperm. Data shown includes LS Means, and Tukey Contrast SE and P-values at 8 and 24 h after incubation at 18°C. Differences were considered significant at $P < 0.05$ (Bold and underlined values), n = 10.

Value	Time	LS Means		Tukey Contrast		
		Conventional	SexedULTRA™	Change	SE	P
Visual Motile	08 h	47.1	51.3	4.2	2.5	0.111
	24 h	41.3	48.0	6.7	2.5	**0.014**
Total Motile	08 h	46.3	48.9	2.7	2.6	0.312
	24 h	37.0	42.0	5.0	2.6	0.063
Prog. Motile	08 h	28.4	30.0	1.6	2.3	0.493
	24 h	18.2	21.6	3.4	2.3	0.146
VIA	08 h	53.6	52.5	−1.1	3.0	0.719
	24 h	48.7	49.7	1.0	3.0	0.752
PIA	08 h	74.2	77.6	3.4	2.6	0.206
	24 h	69.9	76.4	6.5	2.6	**0.020**

4. Discussion

During the three decades since sex-preselection of offspring was proven possible [1,3], the most-quoted issues are the speed at which sperm cells can be separated, the decreased sperm quality and the lower fertility due to the numerous steps and manipulations they undergo during the sex-sorting process. While conventional semen has minimal processing steps, sex-sorted semen passes through over 21 processing steps before cryopreservation [10]. Each one of these steps entails a mechanical, physical and biochemical stress on the sperm cell. Motility, velocity, amplitude of lateral head displacement and membrane integrity assessments of sex-sorted sperm have pointed towards some sperm damage due to the sorting process [5,11]. Functional studies *in vitro* found sex-sorted-related modifications similar to those occurring after *in vitro* capacitation (CTC analysis and protein tyrosine phosphorylation) [12]. DNA fragmentation studies have proven a reduced longevity in sex-sorted compared to non-sorted sperm [13]. Although, there are many reports in literature where the disconnect between *in vitro* sperm parameters and fertility has been demonstrated [14], the lower sperm quality could be one of the factors affecting *in vitro* embryo production and field fertility. Publications report a reduction in blastocyst yields when using sex-sorted compared to non-sorted sperm [15–17], and detailed analysis of developmental kinetics show that the use of sorted spermatozoa in IVF significantly delays the onset of cleavage [18]. Conception rates have also been recognized as an issue since the first breeding trials using sex-sorted sperm took place in rabbits [3] and cattle [19]. Fertility of sex-sorted sperm is known to be 75–80% compared to conventional non-sorted frozen-thawed sperm [20–22]. And the gap between sex-sorted and non-sorted sperm fertility has not been bridged by increasing the numbers of sperm per insemination, which was attributed to a reduction in sperm quality after sex-sorting due to the combined forces of staining, sorting and cryopreservation [23,24].

Large-scale fertility trials over the last three years in New Zealand indicate that sex-sorted fresh sperm at a concentration of 1 million has a relative fertility of around 95% to that of fresh conventional sperm at a concentration of 2 million [25]. It is likely that the process of sex-sorting on its own is not quite so damaging and

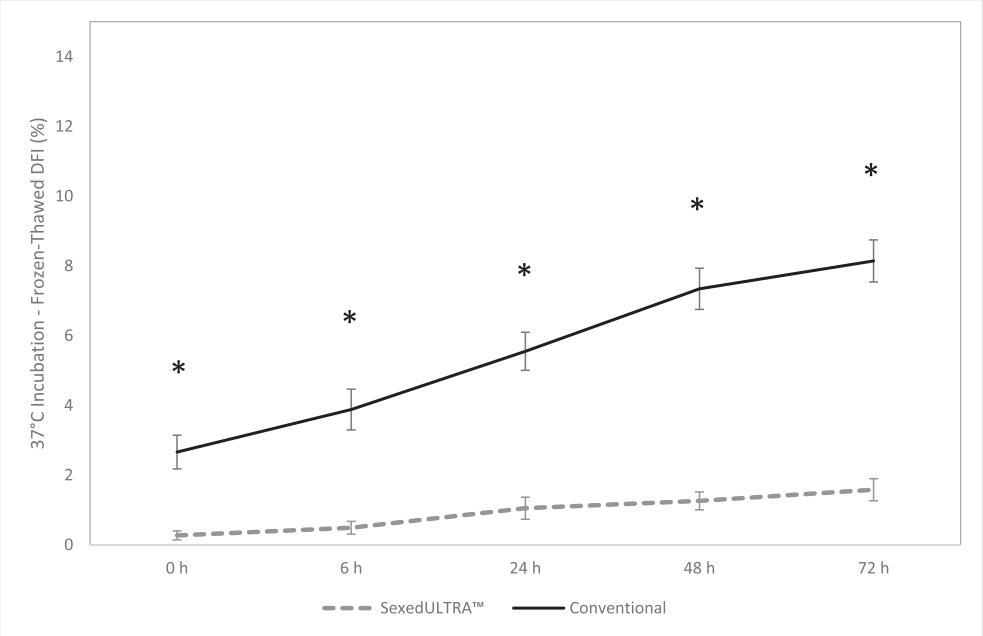

Fig. 1. Comparison of frozen-thawed SexedULTRA™ and conventional percent sperm with fragmented DNA post-thaw (0 h) and 6, 24, 48 and 72 h post-incubation at 37 °C. Differences were considered significant at $P < 0.05$ (*), $n = 10$.

that the more detrimental aspect is the additive effect of cryopreservation after sex-sorting.

A concerted effort has been made in the last several years to improve the biochemistry of media and to provide a less damaging environment that accommodates all the changes that occur during the various steps of processing and sex-sorting before sperm are cryopreserved and stored for artificial insemination. The analytical methods presented in this publication were used to confirm that the separate steps beneficially modified the sorting method resulting in improvements to post-thaw sperm integrity.

This publication reports a significant improvement in the *in vitro* quality of sex-sorted sperm making it comparable to conventional semen levels. Our results show frozen-thawed SexedULTRA™ sperm presents significantly lower DNA fragmentation levels at every time point of the analysis, indicating that longevity is now better than that of non-sorted sperm. All other post-thaw *in vitro* parameters analyzed are equal or better in SexedULTRA™ sperm compared to conventional. Results with fresh sperm are even more clear, where SexedULTRA™ sperm has significantly improved quality at all times of the analysis in DFI, visual and total motility, VIA and PIA compared to non-sorted sperm. This would support the hypothesis that the damage of sex-sorting and cryopreservation is additive, and suggests that the performance of sex-sorted sperm could be improved by finding unique cryopreservation methods for this type of semen.

An important observation in this study was that the *in vitro* sperm quality parameters were enhanced for SexedULTRA™ at each one of the time points of both experiments. Also, across all the sperm characteristics tested, the general decline of sperm quality between 0 h and after the various incubation time points were reduced for SexedULTRA™. In other words, there was an improvement in the ratio of *in vitro* sex-sorted sperm quality post-incubation compared to 0 h. The post-thaw 3 h/0 h ratio was already reasonable for conventional semen (>80%), but not in sex-sorted sperm processed following XY method (60–70%, unpublished results). For SexedULTRA™, the post-thaw 3 h/0 h ratio is now equivalent to that one of conventional semen, which means that sex-sorted sperm are now able to retain their integrity for longer periods of time.

It is also worth noting that the DFI was not high at the baseline (about 2%), but dropped to nearly zero by sex-sorting, indicating that sperm with damaged DNA are removed during the sorting process.

The results presented in this publication are consistent with

Table 2
Comparison of percent visual, total and progressive motility, intact plasma membranes (VIA) and acrosomes (PIA) of fresh SexedULTRA™ and conventional sperm. Data shown includes LS Means, and Tukey Contrast SE and P-values at 0, 12, 24, 48, 72 h after incubation at 18 °C. Differences were considered significant at $P < 0.05$ (Bold and underlined values), $n = 7$.

Value	Time	LS Means		Tukey Contrast		
		Conventional	SexedULTRA™	Change	SE	P
Visual Motile	00 h	75.6	81.4	5.9	2.1	**0.007**
	12 h	71.4	77.9	6.4	2.1	**0.003**
	24 h	69.6	74.9	5.3	2.1	**0.015**
	48 h	64.7	74.3	9.6	2.1	**0.000**
	72 h	63.3	68.3	5.0	2.1	**0.021**
Total Motile	00 h	74.9	82.0	7.1	2.5	**0.007**
	12 h	71.6	79.8	8.2	2.5	**0.002**
	24 h	69.9	76.3	6.3	2.5	**0.015**
	48 h	66.3	75.7	9.5	2.5	**0.000**
	72 h	62.1	67.4	5.2	2.5	**0.042**
Prog. Motile	00 h	54.0	61.3	7.3	3.5	**0.041**
	12 h	51.3	57.4	6.2	3.5	0.082
	24 h	49.7	52.6	2.9	3.5	0.409
	48 h	37.6	44.3	6.6	3.5	0.061
	72 h	28.2	33.2	5.1	3.5	0.151
VIA	00 h	75.8	85.3	9.5	2.9	**0.002**
	12 h	72.4	81.3	8.9	2.9	**0.004**
	24 h	69.6	78.6	9.0	2.9	**0.003**
	48 h	67.8	78.5	10.8	2.9	**0.001**
	72 h	67.2	74.9	7.7	2.9	**0.011**
PIA	00 h	88.7	98.0	9.3	1.2	**0.000**
	12 h	86.9	95.9	9.1	1.2	**0.000**
	24 h	85.3	97.0	11.8	1.2	**0.000**
	48 h	83.5	96.1	12.6	1.2	**0.000**
	72 h	80.1	94.3	14.2	1.2	**0.000**

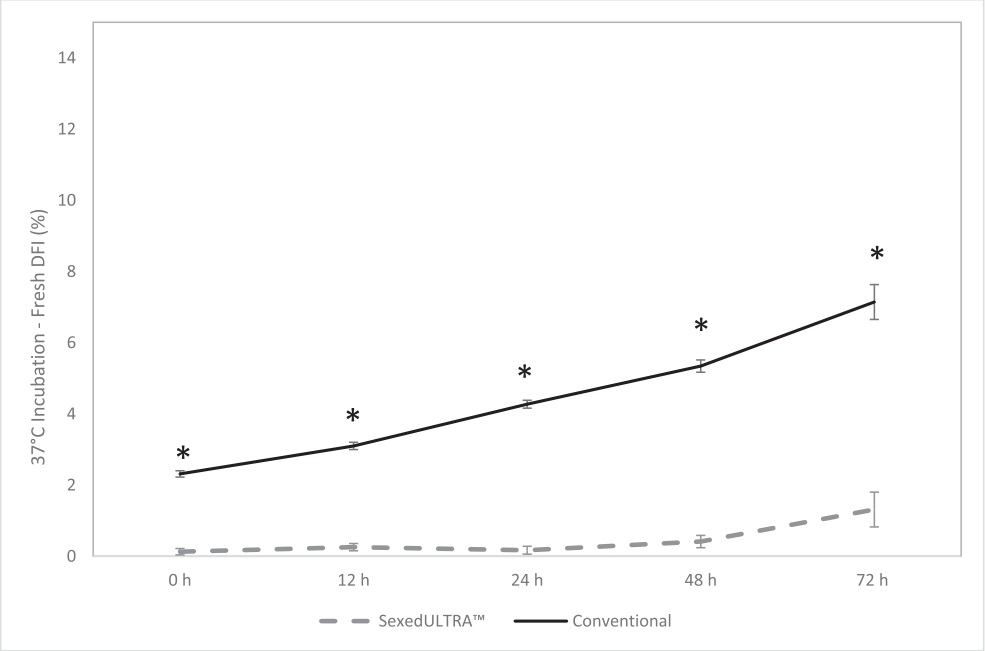

Fig. 2. Comparison of fresh SexedULTRA™ and conventional percent sperm with fragmented DNA after fresh dilution (0 h) and 12, 24, 48 and 72 h post-incubation at 37 °C. Differences were considered significant at $P < 0.05$ (*), $n = 7$.

in vitro fertilization results [26] where total and freezable embryo numbers are significantly higher when using SexedULTRA™ compared with the old method of sperm sex-sorting, demonstrating an improvement in the fertility of sex-sorted bovine sperm. Also, field trials have shown that sperm sex-sorted following the SexedULTRA™ method resulted in a greater conception rates compared to sperm processed following the old sorting method, and that SexedULTRA™ sperm frozen at 4 million sperm per dose presented greater 56 days non-return rates than conventional semen processed at 15 million per dose [27]. When used appropriately, SexedULTRA™ sperm in timed AI programs can display a comparable level of fertility to that observed in naturally cycling animals [28], and relative fertility of SexedULTRA™ compared to conventional semen in conjunction with split-time AI has shown no difference in total pregnancy rates at the end of the 60-day breeding season [29].

This report confirms that some of the issues related to sex-sorting are mitigated by improvements made with the SexedULTRA™ method, which confers a significant benefit in maintaining both fresh and cryopreserved sperm integrity. This sex-sorted sperm quality improvement seems to translate into *in vitro* embryo production and field fertility in a way that, with appropriate times of insemination and with proper synchronization protocols, the gap between SexedULTRA™ and conventional bovine sperm can be greatly reduced if not eliminated.

Acknowledgments

The authors wish to thank Richard W. Lenz for the expertise and support provided through this project.

References

[1] Garner DL, Johnson LA, Lake S, Chaney N, Stephenson D, Pinkel D. Quantification of the X and Y chromosome bearing spermatozoa of domestic animals by flow cytometry. Biol Reprod 1983;28(2):312–21.
[2] Garner DL. Sex-sorting mammalian sperm: concept to application in animals. J Androl 2001;22(4):519–26.
[3] Johnson LA, Flook JP, Hawk HW. Sex pre-selection in rabbits: live births from X and Y sperm separated by DNA and cell sorting. Biol Reprod 1989;41(2):199–203.
[4] Seidel Jr GE. Sexing mammalian sperm where do we go from here? J Reprod Dev 2012;58(5):505–9. Review.
[5] Seidel Jr GE, Garner DL. Current status of sexing mammalian spermatozoa. Reproduction 2002;124(6):733–43.
[6] Hunt K. Sexing technologies: gender vendors in a changing marketplace [cited 2018 Feb 21]. In: The BullVine [Internet]. Available from: http://www.thebullvine.com/technology/sexing-technologies-gender-vendors-changing-marketplace/; 2013 Sept 24.
[7] Sharpe JC, Evans KM. Advances in flow cytometry for sperm sexing. Theriogenology 2009;71(1):4–10.
[8] Evans KM. Interpretation of sex sorting process and new developments. In: Proc 23rd tech conf artif insem and reprod; 2010. p. 93–8.
[9] DeGraaf SP, Leahy T, Vishwanath R. Biological and practical lessons associated with the use of sexed semen. In: Rumin reprod symp proceedings; 2014. p. 125–40.
[10] Vishwanath R, Sedoglavich N, Morad B, Evans M, Sharpe J. Industrial use of sorting flow cytometry in gender pre-selection – lessons learned. In: Intl soc advances cytometry. Annual meeting; 2014. Abstr 34.
[11] Suh TK, Schenk JL, Seidel Jr GE. High pressure flow cytometric sorting damages sperm. Theriogenology 2005;64(5):1035–48.
[12] Bucci D, Galeati G, Tamanini C, Vallorani C, Rodriguez-Gil JE, Spinaci M. Effect of sex sorting on CTC staining, actin cytoskeleton and tyrosine phosphorylation in bull and boar spermatozoa. Theriogenology 2012;77(6):1206–16.
[13] Gosálvez J, Ramirez MA, López-Fernández C, Crespo F, Evans KM, Kjelland ME, Moreno JF. Sex-sorted bovine spermatozoa and DNA damage: II. Dynamic features. Theriogenology 2011;75(2):206–11.
[14] Vishwanath R, Shannon P. Do sperm cells age? A review of the physiological changes in sperm during storage at ambient temperatures. Reprod Fertil Dev 1997;9(3):321–31.
[15] Merton JS, Haring RM, Stap J, Hoebe RA, Aten JA. Effect of flow cytometrically sorted sperm on success rate of *in vitro* bovine embryo production. Theriogenology 1997;1(47):295.
[16] Lu KH, Cran DG, Seidel Jr GE. In vitro fertilization with flow-cytometrically-sorted bovine sperm. Theriogenology 1999;52(8):1393–405.
[17] Bermejo-Álvarez P, Rizos D, Rath D, Lonergan P, Gutiérrez-Adán A. Can bovine in vitro-matured oocytes selectively process X- or Y-sorted sperm differentially? Biol Reprod 2008;79(4):594–7.
[18] Bermejo-Álvarez P, Lonergan P, Rath D, Gutiérrez-Adan A, Rizos D. Developmental kinetics and gene expression in male and female bovine embryos produced in vitro with sex-sorted spermatozoa. Reprod Fertil Dev 2010;22(2):426–36.
[19] Seidel Jr GE, Schenk JL, Herickhoff LA, Doyle SP, Brink Z, Green RD. Insemination of heifers with sexed sperm. Theriogenology 1999;52(8):1407–20.
[20] Schenk JL, Gran DG, Everett RW, Seidel Jr GE. Pregnancy rates in heifers and cows with cryopreserved sexed sperm. Effects of sperm numbers per

inseminate, sorting pressures and sperm storage before sorting. Theriogenology 2009;71(5):717–28.
[21] DeJarnette JM, Nebel RL, Marshall CE. Evaluating the success of sex-sorted semen in US dairy herds from on farm records. Theriogenology 2009;71(1):49–58.
[22] Norman HD, Hutchison JL, Miller RH. Use of sexed semen and its effect on conception rate, calf sex, dystocia, and stillbirth of Holsteins in the United States. J Dairy Sci 2010;93(8):3880–90.
[23] Frijters ACJ, Mullaart E, Roelofs RGM, van Hoorne RP, Moreno JF, Moreno D, Merton JS. What affects fertility of sexed bull semen more, low sperm dosage or the sorting process? Theriogenology 2009;71(1):64–7.
[24] DeJarnette JM, Leach MA, Nebel RL, Marshall CE, Cleary CR, Moreno JM. Effects of sex sorting and sperm dosage on conception rates of Holstein heifers; is comparable fertility of sex sorted and conventional semen plausible? J Dairy Sci 2011;97(7):3477–83.
[25] Xu ZZ. Application of liquid semen technology improves conception rate of sex-sorted semen in lactating dairy cows. J Dairy Sci 2014;97:7298–304.
[26] Gonzalez-Marin C, Lenz RW, Gilligan TB, Evans KM, Gongora CE, Moreno JF, Vishwanath R. SexedULTRA™, A new method of processing sex sorted bovine sperm improves post-thaw sperm quality and in vitro fertility. Reprod Fertil Dev 2016;29(1):204.
[27] Lenz RW, Gonzalez-Marin C, Gilligan TB, DeJarnette JM, Utt MD, Helser LA, Hasenpusch E, Evans KM, Moreno JF, Vishwanath R. SexedULTRA™, A new method of processing sex-sorted bovine sperm improves conception rates. Reprod Fertil Dev 2016;29(1):203–4.
[28] Macedo GG, MFdS Filho, Sala RV, Mendanha MF, EPdC Filho, Baruselli PS. The use of sex-sorted sperm for reproductive programs in cattle. https://doi.org/10.5772/52180; 2013.
[29] Thomas JM, Locke JWC, Vishwanath R, Hall JB, Ellersieck MR, Smith MF, Patterson DJ. Effective use of SexedULTRA™ sex-sorted semen for timed artificial insemination of beef heifers. Theriogenology 2017;98:88–93.