Analysis of Membrane Integrity, DNA Fragmentation and Mitochondrial Function in Pig Spermatozoa Sorted by Flowcytometer

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ABSTRACT

The objective of this study was to determine the potential hazardous effects of sorting process by flowcytometry on the quality of boar spermatozoa by flowcytometer. Freshly collected boar semen was diluted and divided into two groups; control none sorted and sorted. Sperms in sorted group were processed with flowcytometer for cell sorting with 100 μM nozzle under the 20 psi pressure. Measurements on each parameter were made at two time points, 0 hr (right after sorting) and 24 hr post sorting. Although there was a tendency of lower viability in sorted group than none sorted control group, the percentage of live cells in control (75.83±6.92 & 59.53±10.34) was not significantly different from sorted (59.70±7.37 & 43.97±3.76) at both 0 and 24 hr post sorting. However, sorted sperm showed significantly lower mitochondrial function compared to the control at both 0 h (79.37±3.22 vs. 63.50±10.05) and 24 hr (67.27±3.22 vs. 46.97±5.37) time points (p<0.007). Sperm DNA fragmentation rate was significantly lower in control (22.0±7.04) than that of sorted (32.27±7.49) at 24 hr time point (p<0.0002). Taken together, these data suggested that sorting process by flowcytometer may have influenced sperm motility rather than viability. Also high speed sperm sorting by flowcytometer has significant effects on DNA fragmentation on elapsed time after sorting.

(Key words : Flowcytometry, Sperm sorting, Sperm parameters)

INTRODUCTION

Pre-determining the sex of offspring has been a popular idea among animal scientists for several decades. Stably producing offspring of favored sex and avoiding less favored sex would give a great influence on the industry as higher profit can be guaranteed for animal producers. Single sex production would be particularly advantageous for those species, in which the product of major interest is occurred by either male or female but not by both. For example, female dairy cow is valued higher than male as milk is produced only by female and male deer is much more sought after as males are the one producing highly priced antlers. In mammalian species, the sex of offspring is determined by the presence or absence of Y-chromosome which should be introduced from Y-sperm. Understandably, there have been many attempts to separate X-sperms from Y-sperms, although most of them ended without any success. More recently, a new idea of sorting sperms using powerful flowcytometry technique has emerged and begins to show its presence in the area of animal industry (Garner & Seidel, 2008; Son, 2008). The principle of the technique is quite simple. Flowcytometry is an instrument originally developed to characterize and separate cells based on the differences present among cells (see Shapiro 2003 for review). Application of the technique to sperm cell sorting is based on the same principle. Generally, in most mammalian species, sperms bearing X-chromosome is slightly larger than sperms bearing Y-chromosome and, in some mammalian species, this difference is big enough to be detected and used by flowcytometry for sorting. This sorting technique, once established, will be able to open a totally new market. However despite these potentials, the technique has few drawbacks too. To be properly sorted into X- and Y-sperms by flowcytometry, sperms should be sent to an environment filled with mechanical stresses, including high pressure, electrical shock, and UV laser beam. As sperms are fragile and tend to be damaged easily by diverse causes, detrimental effects of sorting on the quality of sperms need to be fully investigated to enhance the effect of reproductive performance with sex-sorted spermatozoa *in vivo* and *in vitro*. Therefore, in this study, we attempted to reveal the effects of sorting on sperm parameters such as, cell membrane integrity, mitochondrial function, and DNA chromatin structure on elapsed time after sorting.

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MATERIALS AND METHODS

Sperm Preparation

Sperm-rich fraction of ejaculates, collected by gloved-hand technique from three mature boars of proven fertility were extended in equal volumes of Androhep™ (Minitüb, Tiefenbach, Germany) and stored at 17℃ throughout the experimental period.

Sperm Sorting

To evaluate the influence of sorting, sperms were grouped into control and sorted and each of them was subjected to sperm parameter tests. Aliquots of 1 ml of diluted semen from sorted group were stained with Hoechst 33342 (5 μg/ml) for 1 h at 37℃ and subjected to sorting process. For the sperm sorting, we used BD FacsAria™ (Beckton & Dickinson, San Joes, CA), which is set as follow: sheath fluid pressure at 20 psi, stream speed at 6 m/s, and sample flow speed at 20 μl per minute. All the quantitative data obtained were statistically analyzed using BD FacsDiva™ software. Before running samples, drop delay was adjusted for optimizing cell separation using Accudrop bead that passed through a 488 nm blue laser (Coherent Sapphire™). Single sperm droplet was allowed to pass through a 100 μm nozzle by piezo-electric mechanism. The sorting rate was maintained at less than 20,000/sec. Sorted sperms were directly collected into a 15 ml polypropylene tube containing 5 ml Androhep media. A total of 2 million cells were collected on a tube and centrifuged immediately at 600 ×g for 10 minutes. Subsequently, supernatant was discarded and a pellet was resuspended in Androhep media and stored at 17℃ until used. The sorting process was not applied to sperms in control group.

Flowcytometry

To analyze the effects of sorting, each of control and sorted sperms were subjected to viability test, mitochondria function test and DNA fragmentation test. For the viability test, cells were stained with two dyes (dual staining), SYBR-14 (green fluorescence emitted and collected through a 530/30 BP filter) and propidium iodide (PI, red fluorescence emitted and collected through a 610/20 BP filter). SYBR-14 is a membrane-permeant DNA dye and tends to be accumulated in live cells. In contrast, Propidium Iodide (PI) is a dye specifically accumulated in the nucleus of dead cell. Thus, cells emitting green fluorescence were regarded as live cells and cells emitting red fluorescence were regarded as dead cells. In the second experiments, mitochondrial function was analyzed to estimate the motility of sperm which is energized by mitochondria. The quality of mitochondria can be accessed using the difference of potential ($\Delta\Psi$) across mitochondrial membrane. In cells with healthy mitochondria (strong $\Delta\Psi$), the lipophilic cationic dye tend to be accumulated at the inside of mitochondria. To estimate mitochondria function, cells were stained with a lipophilic cationic dye, Rhodamine-123 and a dead cell-detecting PI stain to enhance the contrast. Subsequently, stained cells were characterized using a second flowcytometry. Green fluorescence of Rhodamine-123 that passed through a 575/26 BP filter and red fluorescence of PI that passed through 610/20 BP were measured and compared. The percentage of sperms containing fragmented DNA was determined by sperm chromatin structure assay (SCSA; Boe-Hansen *et al.*, 2005). Acridine Orange (AO) dye was used to stain both normal double stranded DNA (green fluorescence emitted and collected by 530/30 BP filter) and damaged single stranded DNA (red fluorescence emitted and collected by 610/20 BP filter). Small particles and debris from semen sample were eliminated using threshold function to minimize the spillover of fluorescence signal.

Statistical Analysis

Paired Student *t*-test was applied for the comparison of group mean differences. In all studies, statistical significance was determined at $p<0.05$.

RESULTS

In the viability test, no significant difference between control and sorted groups were detected at both 0 (75.83±6.92 vs. 59.70±7.37) and 24 hr (59.53±10.04 vs. 43.97±3.36) time points ($p>0.14$, Fig. 1). Mitochondrial function in sorted group (63.50±10.05) was decreased by sorting process compared to the control group (79.37±3.22) right after sorting (0 hr, $p<0.007$). However there was no significant difference between the two groups (67.27±4.14 vs. 46.97±5.37, control and sorted, respectively) at 24 hr time point (Fig. 2, $p>0.126$), indicating that impaired sperm motility may be partially repaired from mechanical damage. The percent of sperm DNA fragmentation in sorted group was significantly higher than that of non-sorted control group at 24 h time point (32.27±7.49 vs. 26.90±7.53, $p<0.0002$, Fig. 3). Although, DNA fragmentation was not statistically different at 0 hr time point, there was a clear tendency of DNA damage being increased in sorted group (22.0 ±0.04 vs. 14.77± 5.48, sorted vs. control).

DISCUSSIONS

Sorting cells by commercial high speed flowcytometer has been employed in diverse areas of medical

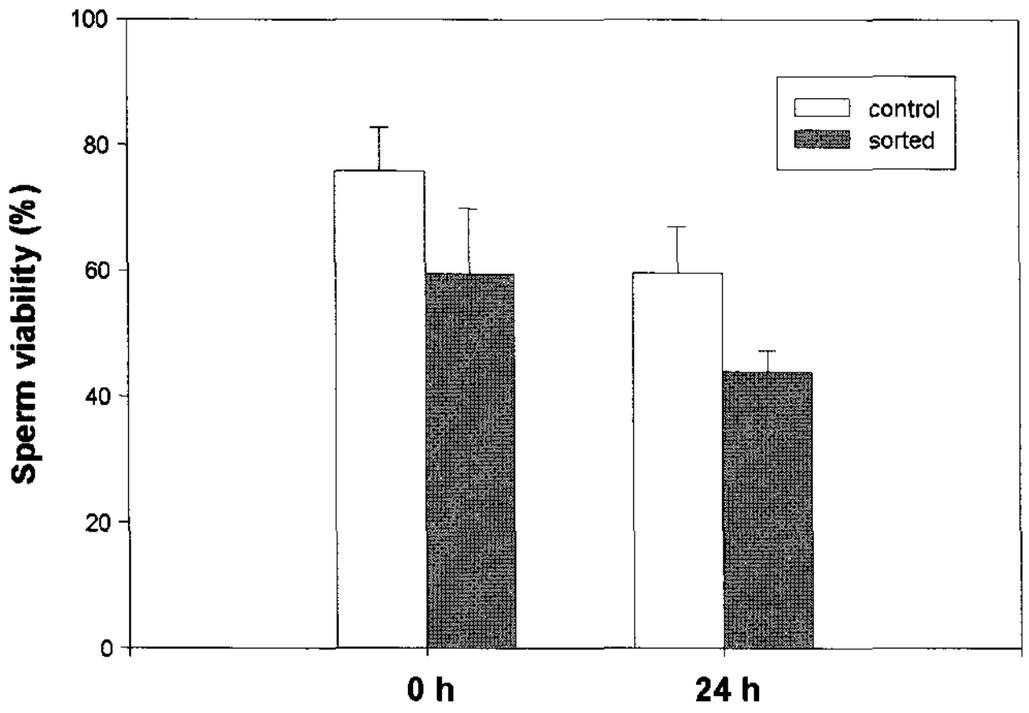

Fig. 1. The percent of live sperm measured by SYBR-14 and propidium iodide in control non-sorted and sorted group at 0 and 24 hr time points.

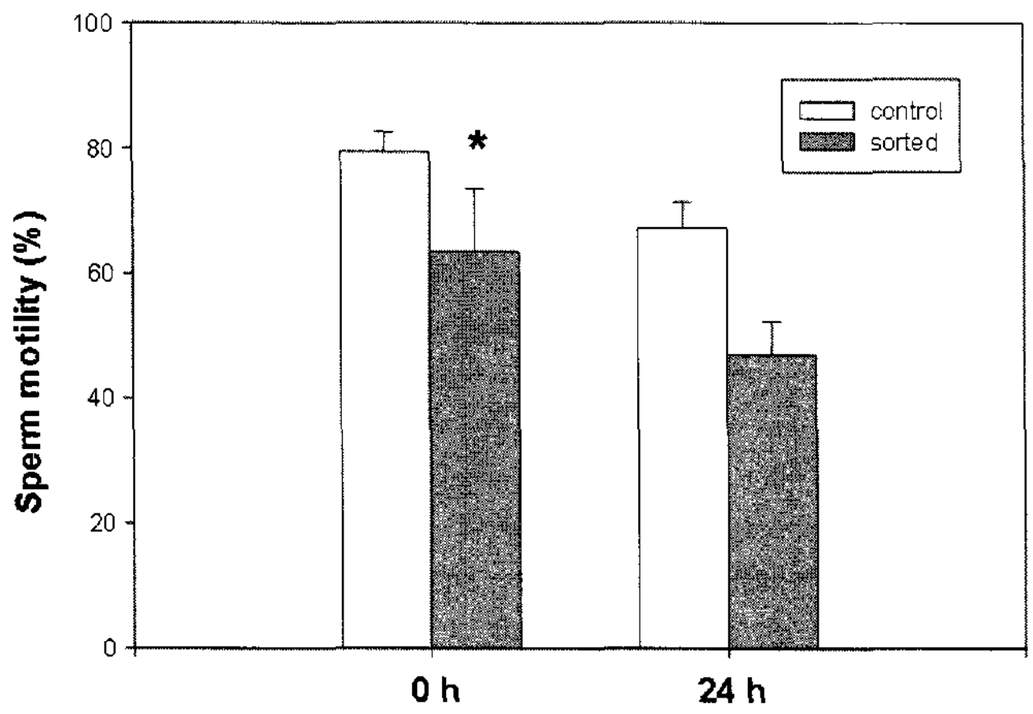

Fig. 2. The percentage of sperm with normal mitochondrial function estimated using Rhodamin-123 and PI in both control non-sorted and sorted groups at 0 and 24 hr time points. * Indicates that statistically significant.

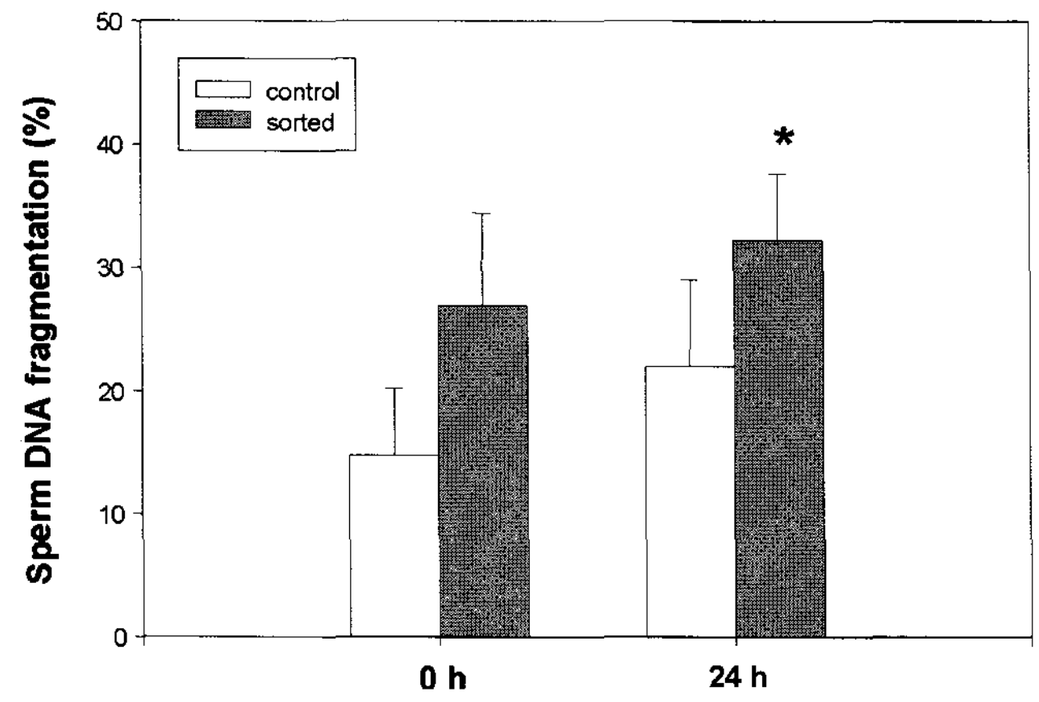

Fig. 3. The percentage of sperm DNA fragmentation measured by sperm chromatin structure assay in control non-sorted and sorted groups at 0 and 24 hr post sorting. * Indicates that statistically significant.

diagnosis and biotechnology and recently expended to the area of sperm sorting. However, due to the fragility of sperm, the quality of sorted sperm is still under questions. Sperm viability test was developed by using the combined usage of two stains, SYBR-14 and PI that identifies both living and dead sperm populations in boar semen simultaneously (Garner and Johnson, 1995). Previously, high pressure applied on cells during high speed sorting was suggested to be hazardous to spermatozoa, especially, to cell membrane (Suh *et al.*, 2005). Interestingly, in the present experiment, we found that high speed sperm sorting affected more significantly the sperm motility than viability (Fig. 1 & 2). Dual staining with Rhodamin-123 and PI would be able to analyze sperm mitochondrial function (Liu *et al.*, 2007). Spinaci *et al.*, (2005) reported that mitochondrial function, estimated with membrane potential, was significantly affected by sorting process. In our experiment, mitochondrial function in sorted group was significantly lower ($p<0.01$) than that of control group at 0 hr, but no difference was found at 24 h time point. We speculated that sperm could regain their motility to a certain degree by unknown endogenous repairing system as time goes and also we could not point out the variations among samples that contributed to large standard errors, therefore no statistical difference. In boar sperm, it has been known that sorting procedure increased the percentage of sperm containing fragmented DNA (De Ambrogi *et al.*, 2006). In agreement, our data showed that sorting procedure affected sperm DNA fragmentation at 0 hr time point and that the damage was even greater at 24 hr ($p<0.05$). Although blastocyst development rate was not affected by sperm sorting in other study (Spinaci *et al.*, 2005), sperm motility and DNA fragmentation obviously were affected by high speed sorting in this study. As sperm DNA damage and weak motility are clearly associated with male infertility (D'Occhio *et al.*, 2007), further investigations are necessary to confirm the relationship between mechanical stresses of sorting and their effects on the quality of sperms.

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