Impact of different dilution techniques on boar sperm quality and sperm distribution of the extended ejaculate

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ABSTRACT

The dilution of ejaculates is a fundamental step for the production of liquid-preserved boar semen. For a long time, it has been recommended to add the extender to the ejaculate. The aim of the present study was to first compare the effect of the position ('center' *vs.* 'wall') where the extender is added to the semen-mixing cylinder (height 32.5 cm; diameter 12.7 cm) using an automatic dispenser (n = 11). In experiment 2 (n = 30), we analyzed the two main dilution methods (extender to the semen ('control') *vs.* 'reverse'). Experiment 3 was carried out to study the dilution effect on kinematics. In Experiments 1 and 2, the sperm distribution 10 min after the dilution and the sperm quality parameters during long-term storage (d1, d3, d5, and d7) were evaluated. In Experiment 3, sperm quality was assessed during short-term storage at 0, 10, 20, 30 and 60 min after semen dilution ('control' *vs.* 'reverse'; n = 6). There were no significant differences ($P > 0.05$) between the treatments in the specific response to bicarbonate, mitochondrial activity, membrane status, thermo-resistance or sperm motility immediately after dilution or long-term storage. The sperm distribution was significantly ($P = 0.029$) affected by the dilution method in Experiment 2. In summary, treatment with the extender first, which is used by only a few European boar studs, leads to comparable results in sperm quality during storage and better results in sperm distribution after dilution. This procedure is also less time consuming, less foam formation occurs during the semen dilution and the procedure is more hygienic.

1. Introduction

High quality extended boar semen is crucial for the success of artificial insemination (AI). Currently, extended semen to be used for AI in pigs is typically stored in 80 to 100 mL doses for up to 5 days at 16–18 °C (Riesenbeck, 2011). As a common practice, raw semen is diluted with the aim to extend the longevity of the spermatozoa and increase the usability of boars of high genetic value to more than 50 semen doses/ejaculate. Increased requirements for semen quality while simultaneously decreasing the number of spermatozoa per AI dose are challenging sperm processing procedures in AI stations. This is due to a vulnerability of sperm caused by higher dilution rates (Centurion et al., 2003) and the need to assure an ideal temperature/time regimen (Schulze et al., 2013a) in the setting of high speed automatized semen dilution and filling processes. Overall, the aim is to minimize the 'dilution effect', which

causes the loss of sperm motility and membrane integrity (Johnson et al., 2000) and changes in the lateral organization of lipids and proteins on the sperm surface, leading to membrane destabilization (Leahy and Gadella, 2011). The harmful effect of dilution has been linked to several factors: a) the removal of seminal plasma factors that contribute to the stabilization of the sperm membranes (Centurion et al., 2003), b) the rapidity of dilution (Bamba and Cran, 1988), c) the use of chemically or physically imbalanced extender media (Dziuk, 1958; Ashworth et al., 1994), d) the cold shock resulting from hypothermic extenders (Schulze et al., 2013a), and e) the physical stress associated with the dilution procedure (Leahy and Gadella, 2011).

Since the pioneering work performed by Milovanov (1934) on the dilution effect, it has always been recommended that the extender should be added in small quantities to the ejaculate, while gently mixing − and not in the reverse order (Götze, 1949). It is common knowledge that the addition of extender to semen pre-laid in a vessel causes a less sudden change to spermatozoa and therefore is less harmful compared to the addition of semen to an extender. However, in laboratories at AI centers, large volumes of extenders (approximately 1.000–7.000 mL for a single ejaculate) will be added to raw semen (200–500 mL), causing foam formation and hygienic risks if the filling nozzle comes into contact with the foam. Foam formation is especially pronounced in extenders containing BSA, but also occurs with other extender media and should be avoided. From the practicable and hygienic perspective, therefore, the addition of semen to extender would be favorable. Reports proving the advantage of the standard dilution procedure for the quality of liquid preserved boar spermatozoa are lacking. The aim of the present study was to compare the effect of the two dilution methods, extender to semen ('control') vs. semen to extender ('reverse'), on sperm quality parameters and sperm distribution in the extended semen. In addition, the effect of the position ('center' vs. 'wall') where the extender is filled into the semen-mixing cylinder was studied. Trials were performed under conditions of semen processing in AI centers, and sensitive methods were used to detect subtle effects on sperm quality.

2. Materials and methods

2.1. Chemicals

All chemicals used in this study were of analytical grade. Unless stated otherwise, they were purchased from Merck (Darmstadt, Germany) and Roth (Karlsruhe, Germany). Propidium iodide (PI) and rhodamine 123 (R123) were obtained from Sigma-Aldrich (Steinheim, Germany), whereas fluorescein-isothiocyanate conjugated peanut agglutinin (FITC-PNA) and *Pisum sativum* agglutinin (FITC-PSA) were purchased from Axxora (Lörrach, Germany).

2.2. Semen collection and selection criteria

The average age (mean ± SD) of the Pietrain boars (n = 47) used in this study was 19.4 ± 4.1 months. All boars were routinely used for the production of AI doses, received commercial feed (pellets) for AI boars and were housed in individual pens equipped with nipple drinkers according to the European Commission Directive for Pig Welfare in one boar stud in Germany. Protocols were carried out according to the general guidelines for semen processing used in AI studs participating in a quality control audit of the Institute for the Reproduction of Farm Animals Schönow (Riesenbeck et al., 2015).

Ejaculates were collected randomly by the gloved-hand method. The gel fraction of the semen was removed by gauze filtration. Only ejaculates that passed minimum requirements for commercial use in AI were included. Criteria for the selection of ejaculates stipulated a minimum of 75% morphologically normal spermatozoa, total sperm motility of at least 70%, and a total amount of $\geq 30 \times 10^9$ spermatozoa per ejaculate.

2.3. Experiment 1

In Experiment 1, the impact of the position of the filling nozzle ('center' vs. 'wall') on sperm distribution after dilution and sperm quality during long-term storage was evaluated in a split-sample procedure. Normospermic ejaculates from 11 fertile Pietrain boars were used. For semen dilution, a SmartDispenser L (Minitüb, Tiefenbach, Germany) with an average pump velocity of 78 mL s^{-1} and a commonly used semen-mixing cylinder (height 32.5 cm; diameter 12.7 cm; equipped with a 3.5 L semen bag, Minitüb, Germany) were used. The filling nozzle was either positioned centrally over the cylinder so that extender was poured into the middle of the vessel ('center') or peripherally so that the extender was poured alongside the inner wall of the cylinder ('wall'). The sperm concentration was adjusted to 23.5 x 10^6 spermatozoa mL^{-1} using a NucleoCounter SP-100 (Chemometec, Denmark). Corresponding to the routine method in the AI center, the dilution of full ejaculates (total volumes between 1.500 and 3.400 mL) was completed at one − step with an isothermic (32 °C) Beltsville Thawing Solution extender (BTS, Minitüb, Germany). Semen was then filled in 95 mL QuickTip Flexitubes® (Minitüb, Germany) with an automatic filling machine (MiniBSP, Minitüb, Germany). The filling volume was 85 ± 1 mL. Finally, all extended samples were placed in a temperature-controlled box at 21 °C for 90 min and subsequently stored in a temperature-controlled cabinet at 17 °C for seven days.

2.4. Experiment 2

Experiment 2 was carried out to investigate the impact of the dilution sequence, e.g., extender to the semen ('control') vs. semen to extender ('reverse'), on sperm distribution 10 min after dilution and sperm quality during long-term storage. Normospermic ejaculates from 30 fertile Pietrain boars were used. The position of the filling nozzle for the dilution method 'control' was central.