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The "dilution effect" in stallion sperm

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ABSTRACT

Dilution of semen to less than 20 × 10^6 sperm/mL has been reported to decrease sperm quality in multiple species, a phenomenon known as the semen "dilution effect." Critical evaluation of stallion semen diluted to these concentrations, however, has not been reported. This study evaluated sperm motion characteristics (percent total motility [TMOT], percent progressive motility [PMOT], curvilinear velocity [μm/s], and percent straightness) and plasma membrane integrity (percent plasma membrane intact [PMI]) in semen samples diluted to 2.5 × 10^6 sperm/mL with the addition of 0%, 7.5%, or 25% seminal plasma (groups T-2.5/0, T-2.5/7.5, and T-2.5/25, respectively), or after simple dilution to 30 × 10^6 sperm/mL (group T-30), or simple dilution to a ratio of 3:1 (extender:semen; group T-3:1SD). Evaluations were performed immediately after semen collection (T0), and after 24 and 48 hours of cooled storage (T24 and T48, respectively). The PMI and TMOT were the highest in group T-3:1SD at T0. At T24, the PMI in groups T-30, T3:1SD and T3:1/30, and T-2.5/0 were higher than that in the other groups ($P < 0.05$), whereas TMOT in group T-3:1SD was higher ($P < 0.05$) than that in all other groups except T-30. By T48, no difference was detected for PMI among groups T-3:1SD, T-30, and T-2.5/0; for TMOT among groups T-3:1SD, T-30, and T-2.5/0, and T-2.5/7.5 ($P > 0.05$), whereas PMOT was the highest in groups T-2.5/0 and T-2.5/7.5 ($P < 0.05$). These findings revealed that treatments in which semen was diluted to a concentration of 2.5 × 10^6 sperm/mL had lower initial PMI, TMOT, and PMOT, but semen quality did not decline after 24 and 48 hours of cooled storage. In this study, TMOT and PMI in dilute semen were less than those in more concentrated semen at T0. This effect, while significant, was small and less apparent after cooled storage.

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1. Introduction

Semen dilution is a commonly used procedure in many species to enable more accurate assessment of sperm quality and improve sperm longevity. Dilution is particularly important for storage of stallion semen because of commercial use of cool-shipped and frozen-thawed sperm. Historically, the "dilution effect" refers to a dramatic decline in sperm motility resulting from excessive semen dilution. The "dilution effect" has been described for rabbit [1–4], ram [3–6], bull [3,5,7–13], boar [14], dog [15], stallion [16,17], and human [3,18] sperm and generally occurs when raw semen is diluted to less than 20 million sperm/mL [1–6,8,10,12–15,18,19].

Milovanov [20] first described the "dilution effect" when he tested the ability of sperm to resist immobilization

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after exposure of sperm to increasing proportions (i.e., volume of diluent to volume semen) of a 1% sodium chloride solution. The loss of sperm motility was attributed to sodium chloride toxicity. Subsequent researchers suggested other causes including a loss of protective seminal plasma (SP) components [1,3,6,15] and/or intracellular and/or cell surface components from the sperm [1–3,5,8,10,15] resulting in a capacitation-like state and, ultimately, premature acrosome reaction [14].

Others suggested that flagellar undulations of sperm induce fluid motion that distributes enzymes, ions, and other dissolved chemicals and gases among plasma membranes of other sperm in close proximity. They suggested that this effect is most pronounced when sperm are within one body length from each other [21].

Diluent components such as SP may enhance [10,18,19,22] or reduce [2,5,6,10,13–15] the dilution effect depending on its concentration. However, other studies report no effect of SP concentration on the "dilution effect" but rather suggest that the final sperm concentration alone is the primary factor involved [7,12,13,18,19]. In addition, diluent type, such as isotonic diluents [6] or high molecular weight additives such as egg yolk [8], milk [19], and serum proteins [4,23], may also play a role.

Varner et al. [19] evaluated the effects of simple dilution of stallion semen with a skim milk-glucose extender at 25, 50, or 100 $\times$ 10^6 sperm/mL. In that study, total sperm motility (TMOT) and progressive sperm motility (PMOT) were higher at 25 than at 50 or 100 $\times$ 10^6/mL beginning after 12 hours of storage. Whereas the lowest sperm concentration (25 $\times$ 10^6/mL) remained higher than that considered necessary for the "dilution effect" in other species (i.e., $< 20 \times 10^6$ sperm/mL); it is notable that sperm quality improved, rather than declined, as dilution increased in that study.

Previous studies revealed either no difference [16] or a reduction [16,17] in fertility when mares were bred with semen of low sperm concentration compared with high sperm concentration. Whereas sperm quality was not compared in these studies, the "dilution effect" may have reduced the sperm quality. Other possibilities suggested by the authors included retrograde sperm loss due to expulsion of the large-volume inseminations resulting in an effectively lower sperm dose remaining within the tubular tract, or an adverse effect on the endometrium (i.e., endometritis) that might occur when large volumes of semen are placed within the uterus. This latter possibility was later disproven [24].

Recently, Hayden et al. [25] used low-volume/dose insemination to compare pregnancy rates achieved with hysteroscopic or transrectally guided insemination techniques using dilute (2.5 or 5.0 $\times$ 10^6/mL) semen. Whereas pregnancy rates using the 5.0 $\times$ 10^6/mL samples were higher than those using the 2.5 $\times$ 10^6/mL samples, total sperm numbers in inseminates were also doubled (1 vs. 0.5 million, respectively). Nevertheless, the mare fertility with 5.0 $\times$ 10^6/mL samples was high (75% pregnancy rate per cycle) suggesting that any "dilution effect" in this study did not adversely affect fertility.

The objective of this study was to determine if a "dilution effect" could be reported in stallion semen diluted in a commercially available semen extender.

2. Materials and methods

Three ejaculates were collected once daily from each of three stallions (two 20-year-old American Quarter Horses and one 9-year-old American Paint Horse) for a total of nine ejaculates. The study was conducted in Southeast Texas. All experimental procedures were performed according to the United States Government Principles for Utilization and Care of Vertebrate Animals Used in Testing, Research and Training and were approved by the Laboratory Animal Care Committee at Texas A&M University.

2.1. Semen collection

Semen was collected using a Missouri model artificial vagina (Nasco, Fort Atkinson, WI, USA) lightly lubricated with 3 to 5 mL of sterile, nonspermicidal lubricating jelly (Priority Care; First Priority, Inc., Elgin, IL, USA) and fitted with an in-line nylon gel filter (Animal Reproduction Systems, Chino, CA, USA). A mare exhibiting behavioral estrus was used for sexual stimulation, and a breeding phantom was used as a mount source. Immediately before semen collection, the stallion's erect penis was cleansed with warm water and thoroughly dried.

2.2. Semen processing

Immediately after semen collection, the gel filter was removed from the collection bottle and the gel-free semen was placed in a 37 °C incubator. Sperm concentration was estimated using the NucleoCounter SP-100 (ChemoMetec A/S, Allerød, Denmark). Prewarmed (37 °C) INRA 96 (IMV Technologies, L'Aigle, France) extender supplemented with TIMENTIN (1 mg/mL; GlaxoSmithKline, Research Triangle Park, NC, USA), hereafter referred to as INRA-T, was added to neat semen to create the following treatment groups: (1) T-3:1SD, simple dilution at a 3:1 (extender:semen) ratio; (2) T-30, extender added to achieve a final sperm concentration of 30 $\times$ 10^6/mL; (3) T-2.5/0, semen extended to 2.5 $\times$ 10^6 sperm/mL with extender containing 0% SP; (4) T-2.5/7.5, semen extended to 2.5 $\times$ 10^6 sperm/mL with extender containing 7.5% SP; and (5) T-2.5/25, semen extended to 2.5 $\times$ 10^6 sperm/mL with extender containing 25% SP. Treatments diluted to 2.5 $\times$ 10^6 sperm/mL (T-2.5/0, T-2.5/7.5, T-2.5/25) were made by diluting 3:1 extended semen with INRA-T, INRA-T supplemented with 7.5% frozen-thawed sperm-free SP (v:v), or INRA-T supplemented with 25% frozen-thawed sperm-free SP (v:v); thus, a total of three treatments were evaluated at a concentration of 2.5 $\times$ 10^6 sperm/mL.

After 24 and 48 hours of cooled storage, an additional treatment (T-3:1/30) was made to facilitate sperm motion analysis by diluting the T-3:1SD to 30 $\times$ 10^6 sperm/mL with extender supplemented with 25% frozen-thawed sperm-free SP (v:v). If T-3:1SD was 30 $\times$ 10^6 sperm/mL or less, no additional dilution was performed before sperm motion analysis. The total sperm and nonintact plasma membrane sperm concentrations were determined for all semen samples at all intervals using a NucleoCounter SP-100.

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