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Original Research

Level of Glycolyzable Substrates in Stallion Semen: Effect of Ejaculation Frequency on Sperm Survival after Cool Storage during the Nonbreeding Season

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A B S T R A C T

Although seminal characteristics are routinely evaluated in the stallion, the effect of collection schedules and seminal plasma on semen quality during cool storage is not well understood, specifically during the nonbreeding season when cryopreservation of stallion semen is preferentially performed. To address these issues, behavioral characteristics, seminal parameters, and biochemical markers (D-glucose, fructose, and citric acid) were measured in ejaculates (n = 60) obtained during the nonbreeding season. Semen was collected from three stallions, twice a day (1-hour gap between successive collections) and two times in a week. Differences between the means of first and second ejaculates were observed for erection latency ($P < .001$), which was higher in second ejaculates and determined a higher total breeding time ($P < .1$). Variations introduced by the stallion were significant for number of mounts ($P < .05$, in first ejaculates), erection latency ($P < .001$, in second ejaculates), and total breeding time ($P < .001$, in second ejaculates). First and second ejaculates differed significantly for sperm motility and sperm concentration ($P < .001$, higher in first ejaculates) and pH ($P < .01$, higher in second ejaculates). D-glucose was present in seminal plasma at a much higher concentration than fructose ($P < .001$) in both ejaculates. There were no significant stallion-associated differences in sperm vitality and pH in the first and second ejaculates as well as in sperm concentration for the second ejaculates. The effect of seminal plasma on equine sperm survival during cooled storage was analyzed by monitoring sperm motility and cell morphology after conservation in an extender medium with and without seminal plasma. When statistically considering seminal plasma and conservation time simultaneously, it was found that these variables affected acrosomal status and midpiece morphology.

1. Introduction

Despite clear seasonal differences that occur with respect to the quality of fresh and frozen–thawed semen, cryopreservation of stallion semen is usually performed during the nonbreeding season [1,2]. Removal of seminal plasma and equilibration of sperm cells at 4°C before freezing is a standard procedure in the equine system ([3], for review).

The components and function of seminal plasma in stallion semen are still poorly understood [4]. It is a commonly accepted fact that seminal plasma functions as an external source of substrates, with which the energy status of sperm cells is maintained; however, not all sugars or more complex carbohydrates are metabolized by

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mammalian sperm cells [5]. Glucose and fructose are two of the most commonly found monosaccharides in mammalian seminal plasma [6]. At the biochemical level, stallion semen presents some distinct features, when compared with other mammalian species. Glucose is the main glycolyzable sugar in horse seminal plasma, unlike what occurs in bull, ram, or human plasma, where the main equivalent sugar is fructose [7]. However, data related to seminal glucose levels are scarce and conflicting. Boronos [8] used one ejaculate from one stallion and reported a level of 12 mg glucose/100 mL, whereas others mention a level of 0.82 mg/mL [9]. Brooks [10] reported the presence of glucose-6-phosphate in extracts from sperm cells sedimented from seminal plasma samples of boars, bulls, and ram, but this compound was absent in stallions.

The spontaneous sexual behavior of stallions during semen collections, both for artificial insemination or semen freezing, is conditioned by season [11], management, and handling [12,13], which sometimes determine the proper attainment of a second ejaculate. The effect of collection schedules and seminal plasma on semen quality during cool storage is not well understood [14-17]. According to Sieme et al. [18], the first ejaculate of double collections (obtained 1 hour apart every 48 hours) had significantly higher sperm concentrations and higher percentages of progressively motile sperm cells after storage at 5°C for 24 hours, with concomitant lower percentages of midpiece alterations than single daily ejaculates. Other authors state that sperm-rich fractions sustain better motilities after cool storage, namely in "poor cooling" male horses [14,15]. Sieme et al. [18] found that the initial sperm quality of the first three jets of the ejaculate was not different from that of total ejaculates and that centrifugation of sperm-rich fractions before freezing improved post-thaw motility and sperm membrane integrity ($P < .05$).

Taken together, our purpose was to: (1) document the levels of energetic substrates in equine seminal plasma after successive ejaculations, (2) determine the effect of successive ejaculations on seminal characteristics, and (3) evaluate semen quality when it is conserved at 4°C without seminal plasma.

2. Materials and Methods

2.1. Semen Collection, Assessment of Sexual Behavior, and Semen Quality

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich (St. Louis, MO). The study was performed during the nonbreeding season (between September and November—decreasing daylight period), and semen samples were obtained from three stallions (8-year-old Garrano, 14-year-old Anglo-Luso, and 15-year-old Anglo-Luso) housed at the Agricultural School, Polytechnic Institute of Coimbra, Portugal, a region with a Mediterranean climate. Stallions were sexually rested during August; therefore, before the start of the experiment, stallions were subjected to daily semen collection for 5 days to start frequent semen collections at a level of daily sperm output.

Semen was collected twice a day (1-hour gap between successive collections), using a phantom (Hannover model) and an artificial vagina (Hannover model), two times a week. Twenty ejaculates per stallion ($n = 60$ ejaculates) were used in this study and sperm quality was assessed as described previously [19]. Seminal traits measured included total volume of ejaculate (TotVol, measured in a glass graduated cylinder), gel-free volume (SemenVol, measured in a glass graduated cylinder after filtration of the raw ejaculate through sterile gauze to remove the gel), gel volume (GelVol, determined by TotVol minus SemenVol), sperm concentration (hemocytometer—Neubauer chamber—and colorimetric methods—Ciba-Corning colorimeter 254, OSI, France, $\lambda = 546$ nm), motility (determined visually under a bright-field microscope [100×, Laborlux, Leica Microsystems GmbH, Wetzlar, Germany] at 35°C on a heated stage), morphology (monitored in semen slides using India ink as a contrasting stain [20]), and intact membrane (evaluated in semen slides using the eosin−nigrosin stain, according to Bloom [21]). Acrosomal status was assessed with SPERMAC (Minitub, GmbH, Germany), according to the manufacturers' instructions. The occurrence and the latency of sexual behavioral events were recorded, as described by Noue et al. [13], except for dismount latency, which was not quantified. Briefly, reference time zero was chosen as the time when the stallion arrived in the collection room. Erection latency (ErecL) was measured from the reference time to erection, total breeding time (TotBT) was measured from the reference time to dismount, and the number of mounts leading to ejaculation (Mounts) was counted as the number of times the stallion jump the phantom with an intromission of the penis in the artificial vagina without ejaculation.

2.2. Fructose, D-Glucose, and Citric Acid

These three seminal markers were measured in seminal plasma using spectrophotometry. Fresh seminal plasma was obtained by centrifuging the ejaculate at 600 × *g* for 5 minutes. Fructose concentrations were determined in the seminal plasma using the resorcinol method [22]. Briefly, 0.1 mL of fresh seminal plasma samples was mixed with 2.9 mL of distilled water. Then, 0.5 mL of NaOH (0.5N) and 0.5 mL of $ZnSO_4$ (10%) were added, mixed, and samples boiled for 1 minute to remove seminal proteins. After centrifugation at 600 × *g* for 5 minutes, 2 mL of the supernatant was collected for determination of fructose levels. Then, 2 mL of resorcinol solution (0.1%) and 6 mL of HCl (30%) were added into tubes and maintained at 90°C for 10 minutes. Absorbance values were read at 520 nm against blanks. A set of fructose standards ranging from 0.0 to 36 mg/100 mL was prepared and used to generate a standard curve.

The concentration of D-glucose in seminal plasma was assessed using the 3,5-dinitrosalicylic acid method [23]. In brief, 1 mL of seminal plasma was mixed with 2 mL of distilled water. Then, 1 mL of 1% 3,5-dinitrosalicylic acid was added, mixed, and boiled for 5 minutes. Absorbance values were read at 540 nm against blanks. A set of glucose standards ranging from 0.0 to 36 mg/100 mL was prepared and used to generate a standard curve.

The citric acid levels in seminal plasma were determined by the method described by Marier and Boulet [24]. Briefly, 200 μL of fresh seminal plasma was mixed with 1.8 mL of 10% trichloroacetic acid solution and boiled for 1 minute to remove seminal proteins. After centrifugation