Theriogenology 86 (2016) 1294–1298

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

The effects of urine concentration, and cushion centrifugation to remove urine, on the quality of cool-stored stallion sperm

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ARTICLE INFO

Article history:
Received 5 February 2016
Received in revised form 20 April 2016
Accepted 20 April 2016

Keywords:
Urospermia
Cushion centrifugation
Stallion

ABSTRACT

Urine-contaminated stallion semen is a clinical problem due to a variety of causes. The effect of the level of urine contamination on the longevity of sperm quality has not been evaluated. The aim of this study was to determine the effects of urine concentration level (0%, 10%, 20%, 30%, and 40%) and cushioned centrifugation and resuspension of the sperm pellet in fresh extender, on measures of sperm quality, immediately after semen collection (T_0), after 1 hour of storage at room temperature (T_1), and after 24 hours of cooled storage (T_{24}). In general, most sperm quality measures declined with increasing urine concentration starting at T_0. Cushioned centrifugation (CC), but not simple dilution, generally maintained sperm quality at T_{24} as compared with T_1. At T_{24}, total sperm motility was higher in all urine-contaminated CC samples compared with uncentrifuged samples ($P < 0.05$); sperm viability was lower in CC than uncentrifuged at a urine concentration of 20%, but higher at 30% and 40% ($P < 0.05$); and DNA quality was decreased (higher % cells outside the main population) in all urine concentrations ($P < 0.05$). Immediate extension in semen extender, followed by cushioned centrifugation and resuspension of the sperm pellet in fresh extender, provided the best option for preserving sperm quality of urospermic semen.

1. Introduction

Urospermia, the presence of urine in the ejaculate, occurs in stallions and can occur sporadically or consistently in stallion ejaculates [1]. Urine contamination of raw semen may result in irreversible damage to sperm, precluding the use of the contaminated semen for use in breeding with either fresh or transported semen. Most cases are idiopathic in nature, thereby limiting therapeutic options. Therapies directed at reducing urine contamination include reducing the amount of urine in the bladder before breeding, pharmacologic treatment to enhance bladder neck closure during ejaculation, or collection of only the sperm-rich portion of the ejaculate using an open-ended artificial vagina [2]. When these treatments are unsuccessful, minimizing the toxic effects of urine on sperm is attempted.

Urine added to raw semen has been shown to be detrimental to the sperm quality of stallions [3]. Addition of a milk-based extender to urine-contaminated semen reduced the detrimental effects of urine on sperm motility. However, centrifugation (without cushion) and resuspension in extender did not improve motility compared with simple dilution [3]. In that study, semen and urine

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http://dx.doi.org/10.1016/j.theriogenology.2016.04.070

were mixed before addition of extender. Longevity of sperm motility in experimental samples was only evaluated for 1 hour after initial exposure of sperm to urine. Ellerborck et al. [4] recently found a significant reduction in sperm motility before, and after, 24 hours of cooled storage when semen was mixed with varying amounts of urine before dilution in semen extender. Centrifugation/resuspension was not used in that study. In addition, these investigators only reported results for a few measures of sperm quality in that abstract.

Centrifugation of stallion semen is commonly used in breeding programs to increase sperm concentration, reduce seminal plasma concentration, reduce inseminate volume, and maximize the longevity of sperm quality [5]. Cushioned centrifugation (CC) has become commonplace as it allows for a higher centrifugation speed and a higher sperm recovery rate, without concurrent sperm damage [6–8]. The procedure uses iodixanol, a nonionic iodinated liquid compound, which is placed beneath semen to protect sperm against concussive damage during the centrifugation process. When using this procedure, we noted that urinary solids (insoluble fraction) passed through the cushion to reach the bottom of the centrifuge tube and were thus easily separated from the urospermic sample. The soluble components of urine were retained primarily in the supernatant. We hypothesized that CC of urine-contaminated ejaculates would aid in reducing exposure of sperm to urine (i.e., both amount and time) and largely remove insoluble and soluble components of urine, thereby improving sperm quality after cooled storage.

The aim of this study was to determine if the addition of semen extender and processing by CC/resuspension could improve sperm quality (i.e., motion characteristics, viability, and DNA quality), as compared with simple dilution, after initial semen contamination with different levels of urine.

2. Materials and methods

Experimental procedures were approved by the Animal Usage and Care Committee of Texas A&M University.

2.1. Urine collection and processing

Urine was obtained from one stallion using a sterile urinary catheter and aseptic technique. After its collection, urine was stirred thoroughly and then immediately aliquoted into 15-mL conical tubes and stored frozen (−80 °C) until thawed for use. Urinalysis (pH, osmolality, ammonia concentration, urea nitrogen concentration, creatinine, and specific gravity) was performed on prefreeze and postthaw urine samples to evaluate potential effects of the freeze-thaw process on urine composition. No apparent differences in urine composition were detected (Table 1).

Table 1
Urinalysis of prefreeze and postthaw urine samples.

Urine sample	Creatinine (mg/dL)	Ammonia (mg/dL)	BUN (mg/dL)	pH	Osmolality	Specific gravity
Prefreeze	262	166	1808	7.7	1575	1.044
Postthaw	268	191	1865	7.6	1535	1.044

Abbreviation: BUN, blood urea nitrogen.

2.2. Semen collection and processing

Three ejaculates were collected from each of four sexually active stallions using a Missouri-model artificial vagina (Nasco, Fort Atkinson, WI, USA) fitted with an in-line nylon gel filter (Animal Reproduction Systems, Chino, CA, USA). Sperm concentration was evaluated in gel-free semen using a fluorescence-based instrument (NucleoCounter SP-100; ChemoMetec A/S, Allerød, Denmark).

Preliminary studies revealed that addition of urine to semen before addition of extender resulted in a marked reduction in sperm motility (data not shown). Therefore, we chose to mix urine, semen, and extender simultaneously to mimic the procedure of collecting a urospermic ejaculate directly into extender that had been preloaded in the semen receptacle.

After semen collection, gel-free semen was mixed with urine and National Institute for Agricultural Research (INRA) 96 semen extender (IMV Technologies, L'Aigle, France) containing ticarcillin-clavulanic acid (1 mg/mL) to a final sperm concentration of 30×10^6 sperm/mL such that the treatments had final urine concentrations of 0%, 10%, 20%, 30%, and 40% urine (v/v). The semen extender was first placed in a 50-mL conical tube (Corning Life Sciences, Lowell, MA, USA). Then the volumes of urine and semen needed to obtain the final sperm and urine concentrations were added to the extender simultaneously.

For uncentrifuged treatments (UC), aliquots of extended semen were placed in 1.5-mL snap cap tubes for later analysis. For CC treatments, 3 mL of a cushion solution (Cushion Fluid, Minitube, Germany) was layered beneath 30 mL of extended semen [7]. The samples were then centrifuged at $1000 \times g$ for 20 minutes [6]. After centrifugation, the supernatant (containing most of the soluble urine fraction) and cushion fluid (containing the insoluble urine fraction) were aspirated and discarded. The remaining sperm pellet was transferred into a 15-mL conical tube (Corning Life Sciences, Lowell, MA, USA), then resuspended in fresh extender to obtain a final sperm concentration of 30×10^6/mL. Resuspended CC samples were subsequently placed into 1.5-mL snap cap tubes for later analysis.

2.3. Sperm chromatin structure assay

An aliquot of semen from each treatment was snapfrozen on dry ice and stored in a −80 °C freezer until analysis was performed. Sample preparation and processing, as well as flow cytometer adjustments, were performed as previously described [9]. Data are presented as percent of sperm cells outside the main population (% cells outside the main population [COMP]-αt).

2.4. Evaluation of sperm motion characteristics

Sperm motility evaluations were performed after incubation at 37 °C for 15 minutes, using a computer-assisted sperm analyzer (IVOS, version 12.2 L; Hamilton Thorne Biosciences, Beverly, MA, USA) as described previously [7]. Motion characteristics of a minimum of 1000 sperm were analyzed using a disposable counting chamber (Leja 20 mm, two-chamber slide; Leja Products B.V.,