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Original Research Article

The effect of radio electric asymmetric conveyer treatment on sperm parameters of subfertile stallions: A pilot study

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SUMMARY

The Radio Electric Asymmetric Conveyer (REAC) has been mostly applied to treat symptoms related to psychological stress. In the study, we demonstrated the effect of REAC-Veterinary Neuro Psycho Physical Optimization (VNPPO) treatment protocol on sperm parameters of subfertile ($n = 11$) and fertile ($n = 4$) stallions. Subfertile stallions showed a reduced sperm concentration, progressive motility and normal morphology compared to fertile stallions. An increase in progressive sperm motility and quality of sperm morphology was found in subfertile stallions after the REAC-VNPPO treatment. The positive effect of the REAC-VNPPO treatment was visible in a reduced number of reacted or absent acrosomes, nuclei with marginated chromatin and presence of cytoplasmic residues. Thus, we suggest that the REAC-VNPPO treatment for stallions with idiopathic subfertility may enhance the reproductive performance of stallions.

1. Introduction

The evaluation of stallion fertility is economically important because superior sires are used more intensely in artificial insemination (AI) procedures in the horse industry. It would be ideal if a stallion's fertility could be predicted prior to the start of his breeding career. Many tests have been used to assay sperm function in order to explore the relationships between sperm parameters and stallion fertility [1–3]. Among the tested parameters, sperm morphology seems to be an important factor for evaluating semen quality [4–6] and a number of different systems are used for its classification in stallion [7]. It is important to consider that the percentage of

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morphologically normal spermatozoa in ejaculates may change in repeated daily collections [8].

Conventional light microscopic examination of semen ejaculate is needed to evaluate sperm concentration and motility, yet it does not fully provide potential indicators of functional impairment in sperm morphology [9]. Transmission electron microscopy (TEM) can be considered the only tool able to evaluate fine sperm structure. TEM has been used to characterize human sperm alterations caused by different pathologies, such as anatomical problems, genitor-urinary infections, hormonal imbalance, chromosomal alterations or gene anomalies [10]. TEM is a suitable technique for advanced fertility diagnosis in stallions, offering a connection between fertility and sperm morphology [11].

Life stress in humans has been hypothesized to alter the dynamic regulation of the autonomic, neuroendocrine, and immune systems [12]. The effect of stress on reproduction has been reported in rats [13] and pigs [14], and was found to critically depend on the timing of stress, genetic predisposition to stress and the type of stress. Both men and women experience significant infertility-specific psychological distress in the context of *in vitro* fertilization, as revealed by several psychological questionnaires [15]. Even though the estimation of stress in animals is difficult, its effect on pain in horses and incorporating pain scales for equine practice have been reported [16]. Radio Electric Asymmetric Conveyer (REAC; [17,18]) has been applied in clinical settings in many specialized areas for the treatment of illnesses and symptoms most frequently related to psychological stress [19–21]. Recently, we proposed REAC technology as a new medical tool for stress management, applied to human idiopathic male infertility [22]. The REAC treatment protocols are painless and non-invasive, and do not require collaboration of the patient, and no side effects were observed. Moreover, this treatment is not pharmacological and it does not interfere with the concomitant use of other therapies. Our goal was to study the effect of a specific REAC treatment protocol, Veterinary Neuro Psycho Physical Optimization (VNPPO), on the sperm morphology of subfertile stallions and, consequently, its role as a treatment in male equine reproduction.

2. Materials and methods

2.1. Animals and semen collection

The investigation was performed during the 2009 breeding season of eleven stallions (seven healthy subfertile, four healthy and fertile) of different breeds (Thoroughbred, Warmblood and Arabian), and different ages (from 12 to 24 years). The stallions were housed individually in boxes, and fed both good hay ad libitum and 2.5 kg of concentrates twice daily. They had been housed in the Reproduction Center under standard conditions for over five years and used exclusively for breeding, and they are currently still housed at the same center. All evaluated stallions had a career in sports flat racing and show jumping. They did not show any signs of stress-induced behavior. The stallions considered to be subfertile had a reduction of semen parameters (total and progressive motility, concentration) at the time of sampling. The qualitative evaluation of semen was always repeated after 24 and 48 h. Semen samples from all stallions were used for AI, and their fertility rate during the whole breeding season had been above 30% in subfertile stallions and 70% in fertile stallions. The study was performed with the assent of the authorized veterinary control as provided by law (no. 116/92), and the stallions' owners signed an informed consent.

2.2. Experimental design

Semen was collected from March to July 2009 (three times a week) with the use of artificial vagina. After collection, semen samples were filtered through a sterile gauze to remove the gel fraction and debris. Multiple semen parameters were routinely determined, including volume, color, consistency, pH and sperm concentration (Densimeter 590°-Animal Reproduction System, Chino, CA, USA). Total and progressive motility were assessed using a Ceros s/nv 12.0l/Power-Hamilton-Thorne Research image analyzer (HD Scientific Supplies, Wetherill Park, New South Wales, Australia) with an external phase-contrast microscope equipped with camera, a 10× phase-contrast negative objective and a warmed (37 °C) stage. Semen was loaded in a 20-μm depth Leja chamber (Leja, Amsterdam, The Netherlands). Four randomly selected microscopic fields (with a total number of sperm more than 200) were scanned. Thirty frames were captured for analysis of each sperm. Kinematic parameters measured by Computer Assisted Sperm Analyzer (CASA, HD Scientific, Australia) were curvilinear velocity (VCL; μm/s), linear velocity (VSL; μm/s) and the mean trajectory of the spermatozoa per unit time (VAP; μm/s). The software settings were adjusted to obtain a clear identification of motile sperm, defined on the minimum size and contrast, and immotile sperm on static size gates and static intensity gates. The linear (progressive) motility was defined as the percentage of motile sperm with the ratio VSL/VAP greater than 0.75 (straightness threshold = 75%). To compare the fertility rates before and after the treatment, semen samples from all stallions were used for AI on Days 1 and 75.

Sperm morphology was evaluated by transmission electron microscopy (TEM; Philips EM208 TEM (Philips Scientifics, Eindhoven, The Netherlands). Seven subfertile stallions were treated with REAC-VNPPO as described below (Fig. 1). During and after the REAC-VNPPO treatment, semen characteristics were monitored over three months. In the current study, semen samples examined by TEM from each stallion were collected: (1) before the beginning of the treatment (baseline, Day 1), (2) during the treatment: on Day 14, (3) during the treatment: on Day 28, (4) after the treatment: on Day 58, and (5) after the treatment: on Day 118. At these same times, semen samples were recovered from fertile stallions.

2.3. Transmission electron microscopy

Ejaculated semen samples were fixed in cold Karnovsky fixative (Sigma–Aldrich, St. Louis, MO, USA) and maintained at 4 °C for 2 h. Fixed semen was washed in 0.1 mol/l cacodylate buffer (pH 7.2) for 12 h, postfixed in buffered 1% osmium tetroxide (Sigma–Aldrich, USA) for 1 h at 4 °C, dehydrated and embedded in Epon Araldite (Sigma–Aldrich, USA). Ultra-thin sections, stained with uranyl acetate and lead citrate, were