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Original Research

Is Resveratrol Effective in Protecting Stallion Cooled Semen?

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ABSTRACT

The aim of this work was to evaluate the effect of resveratrol (RSV) during liquid storage of stallion sperm for 24 hours at either 10°C or 4°C. The antioxidant RSV was added to reduce the oxidative damage that occurs during cold storage. Aliquots of 2 mL of diluted semen were stored either at 4°C or 10°C under anaerobic conditions, in the absence (control group) or presence of RSV at different concentrations (10, 20, 40, and 80 μM). Sperm quality parameters were assessed at 0 hours and after 24 hours of storage. Resveratrol treatment did not affect sperm quality parameters at 0 hours. At 24-hour storage, a significant ($P < .01$) decrease of sperm quality was observed independently from RSV supplementation and storage temperature. A significant decrease of viable spermatozoa with high mitochondrial membrane potential (SYBR+/PI−/JC-1+) was evident at 24-hour storage in 40- and 80-μM RSV groups compared with control group. Moreover, a decline of total motility in 80-μM RSV group compared with the control group and a decrease of progressive motility and average path velocity in 80-μM RSV group compared with control and 20-μM RSV groups were observed. In conclusion, our findings demonstrate that RSV supplementation does not enhance sperm quality of stallion semen after 24 hours of storage. Moreover, 40- and 80-μM RSV concentrations could damage sperm functional status, probably acting as pro-oxidant. Finally, although 24-hour storage significantly affected most of the sperm quality parameters, no significant differences were found in groups maintained at 4°C or 10°C, suggesting that stallion semen could be equally preserved at these different temperatures.

1. Introduction

The use of cooled shipped semen has become a routine method in the equine industry to extend the life expectancy of the spermatozoa that are transported to breed mares for artificial insemination [1]. Several key factors affect the life span of spermatozoa such as collection protocols, chemical composition of semen extenders, centrifugation, cooling rate, and different storage temperatures [2–4]. During the last few years, new insights into cooled semen technology have been made and opened possibilities to improve semen quality and fertilizing ability. Indeed, many cellular injuries appear during the cooling process, such as the disruption of membrane lipids, resulting in damage to mitochondria and loss of integrity of plasma and acrosomal membranes. These stresses, collectively called "cold shock," result in an irreversible loss of motility, viability, and fertilizing capacity of sperm [5]. The generation of reactive oxygen species (ROS), which occurs during low-temperature storage, is one of the causes of cold shock damage. The oxidative stress, caused by

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abnormal ROS production, leads to damage to membrane phospholipids, chromatin, and proteins [6,7]. The susceptibility of spermatozoa to oxidative damage is attributed to the high concentration of unsaturated fatty acids in membrane phospholipids, the limited antioxidant capacity, and their ability to generate ROS [8,9].

Several antioxidants have been used in extender medium to reduce oxidative damage: ascorbic acid and catalase [10], pyruvate and xanthurenic acid [11], melatonin [12], superoxide dismutase [13], butylated hydroxytoluene [14], and quercetin [15]. Resveratrol is a natural grape-derived polyphenolic phytoalexin that possesses pleiotropic effects including anticancer, antiaging, anti-inflammatory, and antioxidant ones [16,17]. Resveratrol effectively scavenges superoxide and peroxide radicals generated from enzymatic and nonenzymatic systems, and it affords protection against damage caused by ROS in somatic cells [18]. Resveratrol may have reversed effects depending on both dose and species. The antioxidant power of RSV on male gametes has already been tested during conservation of ram, human, and boar semen. According to Sarlós et al [19], treating ram semen (during cooling at 5°C for 24 hours or incubating the samples at 37°C for 2 hours) with 15 or 20 μM of RSV significantly inhibits lipid peroxidation (LPO); moreover, 15 μM of RSV prolongs the conservation period of semen, maintaining motility and slowing down the appearance of acrosomal damage. Recent studies in human demonstrated a reduction of LPO in semen cryopreserved with 0.1, 1, and 10 mM of RSV and in fresh semen incubated for 1 hour at 37°C with 15 μM of RSV [20,21]. Moreover, according to Collodel et al [21], progressive sperm motility increased in fresh semen incubated for 1 hour at 37°C with 30, 15, and 6 μM of RSV, whereas treatment with 100 μM of RSV led to a loss in viability in human spermatozoa and in rat spermatocytes. In the boar, various doses of RSV (10, 33, 66, and 100 μM) supplemented during semen preservation at 17°C for 7 days did not improve sperm quality parameters [22].

Based on this information, the main aim of this work was to test the effects of supplementation of RSV during stallion semen conservation for 24 hours at 4°C or 10°C, temperatures able to maintain good stallion semen fertility [23], and to determine its effects on the main parameters of semen quality: motility, viability, and acrosome reaction (RA); mitochondrial membrane potential; and sperm DNA integrity.

2. Materials and Methods

Unless otherwise stated, all chemicals were purchased from Sigma–Aldrich (Milan, Italy).

All experimental procedures were carried out according to DL 116/92, implementation of the 86/609/EEC, regarding the protection of animals used for experimental or other scientific purposes. Furthermore, the protocol was approved by the University of Bologna Care Committee and the Italian Ministry of Health.

2.1. Experimental Design

Three fresh ejaculates were collected from two Standardbred stallions of proven fertility, individually housed at the National Institute of Artificial Insemination, University of Bologna, Italy, using a Missouri artificial vagina with an inline filter (Nasco, Fort Atkinson, WI). Ejaculates were immediately evaluated for volume, concentration (NucleoCounter SP 100; ChemoMetec, Denmark), and motility (see Section 2.5) and sent to the laboratory within 10 minutes at 20°C–25°C.

Semen was diluted to a final concentration of 30×10^6 cells/mL spermatozoa in Kenney extender [24] and divided in aliquots of 2 mL filling up 2 mL Eppendorf to keep out the presence of air and to guarantee anaerobic conditions. Semen samples were placed in a water bath at room temperature, supplemented with RSV at different concentrations (0, 10, 20, 40, and 80 μM), and then stored in two different refrigerators set to 4°C or 10°C. Sperm quality parameters were assessed immediately after RSV supplementation (0 hours) and after 24 hours of storage at 4°C or 10°C.

2.2. Evaluation of Viability

Twenty-five microliters of semen were incubated with 2 μL of a 300-μM solution of propidium iodide (PI), and 2 μL of a 10-μM solution of SYBR Green-14, obtained from the live and/or dead sperm viability kit (Molecular Probes, Inc, Eugene, OR) for 5 minutes at 37°C in the dark. Ten microliters of the stained suspensions were placed on clean microscope slides, carefully overlaid with coverslips, and at least 200 spermatozoa per sample were observed under a Nikon Eclipse E 600 epifluorescence microscope (Nikon Europe BV, Badhoevedorp, The Netherlands). Spermatozoa stained with SYBR-14 but not with PI were considered as viable (SYBR-14− and PI−). Spermatozoa both SYBR-14+ and PI+ and those SYBR-14−/PI+ were considered with damaged membranes or dead.

2.3. Evaluation of RA

The occurrence of an RA was evaluated using fluorescein isothiocyanate (FITC)-conjugated agglutinin derived from *Pisum sativum* (FITC-PSA) staining. Briefly, the spermatozoa, after washing twice with phosphate buffered saline, were fixed and permeabilized for at least 30 minutes at −20°C in 95% ethanol, dried into microscope slides, incubated with FITC-PSA solution (1 mg of PSA-FITC and/or 10 mL of H_2O) for 15 minutes in the dark, and then observed using the previously described microscope. The presence of a green acrosomal fluorescence was considered indicative of an intact acrosome, whereas a partial or total absence of fluorescence was considered to indicate acrosome disruption or RA.

2.4. Evaluation of Mitochondrial Activity

For each sample, an aliquot (25 μL) of semen was incubated with 2 μL of a 300-μM PI stock solution, 2 μL of a 10-μM SYBR Green-14 stock solution, both obtained from the live and/or dead sperm viability kit (Molecular Probes, Inc), and 2 μL of a 150-μM JC-1 solution for 20 minutes at 37°C in the dark. Ten microliters of the sperm suspension were then placed on a slide and at least 200 spermatozoa per sample were scored using the previously described