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Heavy ion radiation can promote greater motility and enolase protein expression in ram sperm in *in vitro* liquid storage

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ABSTRACT

The aim of the study was to determine the effects of heavy ion radiation (HIR) on ram sperm quality during 24 h of *in vitro* liquid storage at 15 °C, and identify the most appropriate dose which did not injure, but actually improved sperm quality and confirmed the relationship between highly expressed enolase and ram sperm quality during storage *in vitro*. Six Dorset ram (*Ovis aries*) semen pools from five mature and healthy rams were each divided into seven experimental groups with different doses of HIR (0, 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 Gy) under the same experimental conditions. Sperm motility, viability, ATP content, and the gene and protein expression of enolase were measured at 24 h of storage. Irradiated semen which had been stored for 24 h, retained not only greater sperm motility, viability, and ATP content, but had greater enolase protein expression. This was evidenced by increased amounts of mRNA for this enzyme and amount of enolase protein as compared with semen from control rams, especially for the 0.1 Gy group ($P < 0.001$). These results indicate that HIR can promote enhanced motility and viability during *in vitro* liquid storage, and the 0.1 Gy may be a suitable dose for improving sperm quality. Greater amounts of enolase and ATP content may results from enhanced sperm glycolysis by HIR. HIR enhances sperm glycolysis to generate sufficient ATP for maintaining sperm motility during storage.

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1. Introduction

Radiation technology has been widely used in plant genetics and breeding (Wang et al., 2012). Since the discovery of X-rays, it has been shown that sperm are quite resistant to radiation damage (Van Herpen and Rikmenspoel, 1969). Thus, the application of radiation technology to mammalian breeding has been attempted. Previous studies have used X-rays and laser radiation of cattle (Rikmenspoel, 1975) and rabbit sperm (Iaffaldano et al., 2010) to determine related biological effects and attempted to use irradiation technology in mammalian breeding, but the research results have been inconclusive.

Zhang et al. (1999) reported that heavy ions at small doses (0.5, 1, and 2 Gy) improve human sperm motility and the percentage of sperm acrosome reactions, demonstrating that small doses of heavy ions may improve human sperm quality *in vitro*. Heavy ion radiation (HIR) can improve sperm quality *in vitro*, which is of great significance for mammalian breeding. There is, however, limited information available about what dose of HIR is most suitable to enhance sperm quality. Not all HIR doses can

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improve sperm quality. Indeed, excessive doses of HIR can cause sperm damage. Therefore, to improve sperm quality using HIR in mammalian breeding, the key is to determine a suitable radiation dose. In a previous study (He et al., 2013), two-dimensional electrophoresis (2-DE) analysis was used to determine proteomic changes in Dorset ram sperm induced by carbon ion radiation at 0.5 Gy, and it was found that enolase content increased in the irradiated sperm. Because enolase is related to the generation of ATP, there may be a relationship between the greater amounts of enolase and ram sperm quality after HIR. This is, therefore, the question was addressed in the present study.

Limited reference information exists regarding the effects of HIR on ram semen liquid storage *in vitro*. In the present study, effects of different HIR doses on ram sperm motility, viability, ATP content, and amounts of enolase mRNA and protein expression were investigated after 24 h of *in vitro* liquid storage at 15 °C.

2. Materials and methods

2.1. Semen collection, dilution and examination

Semen was randomly collected six times (six pools, $n = 6$) using an artificial vagina from five mature and healthy Dorset ram (*Ovis aries*) under the same breeding conditions during a 4-month period 3- or 4 ejaculates/pool; the sperm concentration and motility of each pool were subsequently evaluated using a counting cell chamber by light microscopy (Nikon 80i; Tokyo, Japan) at 400× magnification (Janetta et al., 2009). Only ejaculates with a wave motion scoring >3 on a scale of 0–5 and with a sperm concentration of $>2.5 \times 10^9$/mL were accepted (O'Hara et al., 2010). Each pool was thoroughly mixed, then mixed with 5× volume of diluent (2.422 g Tris, 1.34 g citric acid, 0.5 g fructose, 500 IU benzylpenicillin, and streptomycin in 100 mL of deionized water; Hafez, 2000; Triwulanningsih et al., 2010). Then the mixture was divided into seven aliquots, protected from light, and incubated at 37 °C water for 30 min in a vacuum cup (the volume was 1.5 L; Wanxiang, China). One sample was not irradiated as a control (0 Gy); the other samples were irradiated with different doses of HIR.

2.2. Doses and irradiation

The experiment was divided into seven dose groups including 0, 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 Gy. Heavy ion beam current was $^{12}C^{6+}$ at 250 MeV/U and 31.3 keV μm^{-1} of the beam entrance, which was supplied by the Heavy Ion Research Facility in Lanzhou (HIRFL) at the Institute of Modern Physics, Chinese Academy of Sciences (Lanzhou, China). The dose rate was approximately 0.5 Gy min^{-1}. An air isolation room was used to detect the dose. The acquisition of dose data was auto-matically controlled by a computer. Particle fluence was determined from an air-ionization chamber signal according to the calibration of the detector (PTW-UNIDOS, PTW-Freiburg Co., Wiesbaden, Germany). All groups were irradiated at room temperature and were first placed in 37 °C water in a vacuum cup after irradiation, and were then brought back to the laboratory immediately. After irradiation, all samples were stored at 15 °C for 24 h (Iaffaldano et al., 2010).

2.3. Sperm motility and viability

After 24 h, samples were diluted to 30×10^6 sperm/mL with diluent and sperm motility counts were used a counting cell chamber by light microscope at 400× (Nikon 80i). Percent sperm motility was calculated using the number of motile sperm over the total number of sperm (both motile and non-motile; Yan et al., 2007). Sperm viability was tested using MTT assay. Sperm suspensions (50 μL) were mixed with 25 μL MTT reagent (5 mg/mL in PBS) and incubated in a 96 microplate well at 37 °C for 2 h. After centrifugation and pipetting exhaustively, 100 μL of DMSO dissolved in formazan were added. Sperm activity was estimated as an absorbance at 490 nm (Tecan M200, Switzerland; Ohatni et al., 2004; Li and Zhang, 2013).

2.4. Detection of ATP

The amount of ATP was measured by the protocol of an ATP assay kit (Beyotime, China). Sperm aliquots of 1×10^6 sperm were homogenized with 200 μL of lysis buffer from the ATP assay kit, then vortexed on a shaker for 1–2 s. After centrifugation at $12{,}000 \times g$ for 5 min at 4 °C, the supernatant was transferred to a new tube for ATP determination. Luminescence from a 100 μL sample was assayed in a luminometer together with 100 μL ATP detection buffer from the ATP assay kit. The standard curve of ATP concentration was prepared from a known amount (1 nM to 1 μM; Peng et al., 2009).

2.5. Immunoblotting and immunofluorescence

Sperm were treated with a lysis buffer containing 7 mol/L urea, 2 mol/L thiourea, 4% (W/V) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (Chaps), and 2% (W/V) dithiothreitol (DTT) in the presence of 1% (V/W) protease inhibitor cocktail (Sigma Chemical, St. Louis, MO, USA). Protein concentration was measured by the Bio-Rad Bradford protein assay while bovine serum albumin (Sigma) as a standard. Protein extracted from the testis (60 μg/10 μL) was resolved in SDS-PAGE [12% (W/V) polyacrylamide gel] and then transferred to a PVDF membrane. The membranes were blocked with 5% skimmed milk in Tris buffer saline (TBS), and immunoblotted with the rabbit polyclonal IgG anti-enolase (Cat. #SC-15343) anti-β-tubulin (Cat. #SC-9104; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and a horseradish peroxidase-labeled secondary antibody (Cat. #ZB2301; Beijing Zhongshanjinqiao Biotechnology Co., Beijing, China). Immunoreactivity was detected using an enhanced chemiluminescent HRP substrate kit (Millipore, Billerrica, USA) and the images were captured by a FluorChem 2 imaging system (Alpha Innotech, San Leandro, CA, USA). Quantitative analysis of the relative density of the bands in Western blots was performed using the Quantity One 4.5.2 image analysis software (Bio-Rad). Images were corrected for background and expressed as optical density (OD/mm^2).

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