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Regulation of steroid hormones and energy status with cysteamine and its effect on spermatogenesis

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ABSTRACT

Although it is well known that cysteamine is a potent chemical for treating many diseases including cystinosis and it has many adverse effects, the effect of cysteamine on spermatogenesis is as yet unknown. Therefore the objective of this investigation was to explore the effects of cysteamine on spermatogenesis and the underlying mechanisms. Sheep were treated with vehicle control, 10 mg/kg or 20 mg/kg cysteamine for six months. After that, the semen samples were collected to determine the spermatozoa motility by computer-assisted sperm assay method. Blood samples were collected to detect the levels of hormones and the activity of enzymes. Spermatozoa and testis samples were collected to study the mechanism of cysteamine's actions. It was found that the effects of cysteamine on spermatogenesis were dose dependent. A low dose (10 mg/kg) cysteamine treatment increased ovine spermatozoa motility; however, a higher dose (20 mg/kg) decreased both spermatozoa concentration and motility. This decrease might be due to a reduction in steroid hormone production by the testis, a reduction in energy in the testis and spermatozoa, a disruption in the blood-testis barrier, or a breakdown in the vital signaling pathways involved in spermatogenesis. The inhibitory effects of cysteamine on sheep spermatogenesis may be used to model its effects on young male patients with cystinosis or other diseases that are treated with this drug. Further studies on spermatogenesis that focus on patients treated with cysteamine during the peripubertal stage are warranted.

1. Introduction

Cysteamine (NH_2-CH_2-CH_2-SH, β-mercaptoethylamine) is a naturally existing bioactive substance that modulates the endocrine and metabolic status of animals (Besouw et al., 2013a; Hoagland and Novelli, 1954). However, the plasma concentration of cysteamine in animals or humans is very low (Coloso et al., 2006; Smolin et al., 1998). The major functions of cysteamine include the synthesis and oxidation of fatty acids, the oxidation of pyruvate in the citric acid cycle, and depletion of tissue somatostatin (Szabo and Reichlin, 1981). Cysteamine is the only effective treatment for cystinosis, a lysosomal storage disorder caused by mutations in the gene encoding cystinosin-lysosomal cystine transporter (CTNS) on chromosome 17p3 (Ariceta et al., 2015; Elmonem et al., 2016). Cysteamine can reduce cellular cysteine levels and extend patient life. Recently, the potential of cysteamine to replace previously used drugs has been noted (Besouw et al., 2013a). Cysteamine has been used to treat cystic fibrosis (Devereux et al., 2015; De Stefano and Maiuri, 2015; Charrier et al., 2014), Huntington's disease (Ross and Tabrizi, 2011; Schulte and Littleton, 2011; Shults et al., 1986), Parkinson's disease (PD) (Pillai et al., 2008; Shieh et al., 2008), nonalcoholic fatty liver disease (Cohen et al., 2011), malaria (Min-Oo et al., 2007; Min-Oo et al., 2010), cancer (Bacq et al., 1953; Wan et al., 2011), sickle cell anemia (Hassan et al., 1976), HIV-I (Ho et al., 1995), paracetamol (acetaminophen) hepatotoxicity (Besouw et al., 2013a), and immune diseases (Bryant et al., 1989). It has also been used in livestock production (Balasubramanian and Rho, 2007; Deleuze and Goudet, 2010; Song and Lee, 2007).

Although cysteamine is a potent chemical for treating cystinosis and other diseases, it has many adverse effects. These include ulcers (De Stefano and Maiuri, 2015; Selye and Szabo, 1973), skin, vascular, neurologic, and muscular problems, bone lesions (Besouw et al., 2011), copper deficiency (Besouw et al., 2013b), and developmental toxicity including embryo malformations, intrauterine growth retardation, and fetal death (Jeitner and Lawrence, 2001; Beckman et al., 1998). The effects of cysteamine on spermatogenesis are, as yet, unknown. Therefore, this study

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aimed to explore the effects of cysteamine treatment on ovine spermatogenesis and to underline its mechanisms, starting from the peripubertal period. Sheep were used in this investigation because their large size enabled the easy collection of semen samples, and secondly, cysteamine has been previously used as a diet additive for sheep (Kelly et al., 2008).

Spermatogenesis is an extraordinary complex process, which is regulated by hormonal and paracrine/autocrine factors, genes, and epigenetic regulators (Schlatt and Ehmcke, 2014). It is a multistep process that produces millions of sperm per day. In the Leydig cells, luteinizing hormone (LH) stimulates testosterone (T) production; this is the key endocrine stimulus for spermatogenesis. The other gonadotrophin, follicle-stimulating hormone (FSH), is also important in spermatogenesis and fertility (Neto et al., 2016; Huhtaniemi, 2015; Escott et al., 2014; Fok et al., 2014). Spermatogenesis is also critically dependent on cellular energy status (the ratio between cellular AMP and ATP; Miki, 2007; Garrett et al., 2008). AMP-activated protein kinase (AMPK) is the energy sensor molecule acting as a regulator of energy balance at both cellular and whole body levels (Hardie, 2011; Carling et al., 2012). It has been found that AMPK activity maintains energy levels under ATP-limiting conditions (Hardie, 2011). Therefore current investigation was aimed to explore the effects of cysteamine on spermatogenesis and the underlining mechanisms. Sheep were treated with vehicle control, 10 mg/kg or 20 mg/kg cysteamine for six months. After that, the semen samples were collected to determine the spermatozoa motility by computer-assisted sperm assay (CASA) method according to World Health Organization guidelines (WHO, 2010). Blood samples were collected to detect the levels of hormones and the activity of enzymes. Spermatozoa and testis samples were collected to study the mechanism of cysteamine's actions.

2. Materials and methods

2.1.1. Animals and treatments. The experiment was conducted on pubertal male sheep at Shouguang Hongde Farmer Co., Weifang, China. Sixty crossbred Small-tail Han sheep × North-east fine-wool sheep (age: 2.5 months) were divided into three groups (control, 10, and 20 mg/kg cysteamine treatments). The treatment lasted for 6 months (age: 8.5 months). The sheep were fed a creep diet containing grass, crop straw, and vegetables, in addition to a basal diet (0.5 kg/sheep/day: 40% corn, 10% soybean meal, 25% palm meal, 10% corn starch residue, and 15% wheat bran). Sheep in the cysteamine treatment groups received both the creep diet and basal diet supplemented with a commercial cysteamine feed additive (supplied by Kangdequan Co, Ltd., Hangzhou, China; containing 30% cysteamine hydrochloride with starch and dextrin as carriers for stabilization) at the equivalent of 10 mg or 20 mg pure cysteamine/kg body weight (BW)/day (≥15 mg pure cysteamine/kg BW is used for treating cystinosis; Besouw et al., 2013a). Sheep in the control group were fed the creep and basal diets with a blank carrier (starch and dextrin, equivalent to the weight fed to the cysteamine group). This investigation was carried out in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of Qingdao Agricultural University. BW was recorded every week and tissue samples were collected post mortem. For each organ collected, some tissue was frozen at −80 °C, and some was fixed in 10% neutral formalin and subsequently paraffin embedded. Then, 5-μm sections were prepared and stained with hematoxylin and eosin (H&E). H&E testis sections were reviewed, blind to treatment, for treatment-related differences and pathological changes (Zhao et al., 2012).

2.2. Semen sample collection and evaluation of spermatozoa motility using a computer assisted sperm analysis system

After 5.5 months of treatment (age: 8 months), fresh ejaculates were collected using the gloved hand technique and stored at 17 °C ready for analysis (Hurtado de Llera et al., 2015). Subsequently, spermatozoa motility was assessed using the computer-assisted sperm assay (CASA) method according to World Health Organization guidelines (WHO, 2010). Before analysis, spermatozoa were incubated at 37.5 °C for 30 min and samples were then placed in a pre-warmed counting chamber (MICROPTIC S.L., Barcelona, Spain). A Micropic Sperm Class Analyzer (CASA system) was used, equipped with a 20-fold objective, a camera adaptor (Eclipse E200, Nicon, Japan) and a camera (acA780-75gc, Basler, Germany); it was operated by an SCA sperm class analyzer (MICROPTIC S.L.). The classification of spermatozoa motility was as follows: grade A linear velocity > 22 μm s^{-1}; grade B < 22 μm s^{-1} and curvilinear velocity > 5 μm s^{-1}; grade C curvilinear velocity < 5 μm s^{-1} and grade D immotile spermatozoa (WHO, 2010).

2.3. Routine blood test

Routine blood tests were performed to analyze the effects of cysteamine on blood cells using HEMAVET 950 (Drew Scientific Inc., FL, USA). Total blood samples were analyzed. Briefly, whole blood was collected in EDTA-coated tubes. The instrument was cleaned and set up as the manufacturer's instructions. Then the blood samples were automatically run one by one. Twenty animal samples were analyzed in each treatment group. The results were statistically analyzed by SPSS software.

2.4. Measurement of plasma steroid hormones and growth hormone

Plasma total E, T, FSH, and LH levels were determined using ELISA kits (Nanjing Jiancheng Bioengineering Institute, China) following the manufacturer's instructions. Briefly, plasma total E, T, FSH, or LH was analyzed in duplicates in a 96-well plate, with each well containing 40 μL of blank, standard, or unknown sheep plasma samples. Plasma growth hormone was assayed using an ELISA kit from Nanjing Jiancheng Bioengineering Institute in a 96-well plate, with each well containing 50 μl of growth hormone (GH) standard, blank, or sheep plasma samples (Liu et al., 2016).

2.5. Measurement of plasma AST and ALT

Aspartate aminotransferase (AST/GOT) and alanine aminotransferase (ALT/GPT) in the plasma were determined directly using kits from Nanjing Jiancheng Bioengineering Institute following the manufacturer's instructions. Three samples from each treatment were determined (Liu et al., 2016).

2.6. Western blotting

Testis and spermatozoa samples were lysed in RIPA buffer containing a protease inhibitor cocktail from Sangong Biotech, Ltd. (Shanghai, China). Protein concentration was determined using a BCA kit (Beyotime Institute of Biotechnology, Shanghai, PR China; Zhao et al., 2015). Goat anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Cat #: sc-48166, Santa Cruz Biotechnology, Inc., Dallas, Texas, USA) was used as a loading control. The information for other primary antibodies (Abs) is listed in Table 1. Secondary donkey *anti*-goat Ab (Cat no. A0181) was purchased from Beyotime Institute of Biotechnology, and goat anti-rabbit (Cat no.: A24531) Abs were bought from Novex® by Life Technologies (USA). Fifty micrograms of total protein per sample were loaded onto 10% SDS polyacrylamide electrophoresis gels. The gels were transferred to a polyvinylidene fluoride (PVDF) membrane at 300 mA for 2.5 h at 4 °C. Subsequently, the membranes were blocked with 5% bovine serum albumin (BSA) for 1 h at room temperature (RT), followed by three washes with 0.1% Tween-20 in TBS (TBST). The membranes were incubated with primary Abs diluted at 1:500 in TBST with 1% BSA overnight at 4 °C. After three washes with TBST, the blots were incubated with the HRP-labeled secondary goat