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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

Table 1
Primary antibody information.

Gene symbol	Name	Cat. #	Predicted size	Source (Animal)	Company
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	sc-48166	37kd	Goat (polyclonal)	Santa Cruz Biotechnology, Inc.
Bax	BCL2-Associated X	bs-4564R	21kd	Rabbit (polyclonal)	Beijing Biosynthesis Biotechnology CO.
Caspas 8	Caspas 8	bs-0052R	12/55kd	Rabbit (polyclonal)	Beijing Biosynthesis Biotechnology CO.
Caspas 3	Caspas 3	bs-0081R	28kd	Rabbit (polyclonal)	Beijing Biosynthesis Biotechnology CO.
Bcl-xl	Bcl-xl	bs-1336R	26kd	Rabbit (polyclonal)	Beijing Biosynthesis Biotechnology CO.
Bcl-2	Bcl-2	bs-4563R	26kd	Rabbit (polyclonal)	Beijing Biosynthesis Biotechnology CO.
SOD	Super oxide dismutase	bs-1080R	22kd	Rabbit (polyclonal)	Beijing Biosynthesis Biotechnology CO.
GPX	Glutathine peroxidase	bs-3882R	22kd	Rabbit (polyclonal)	Beijing Biosynthesis Biotechnology CO.
Catalase	Catalase	bs-2302R	58kd	Rabbit (polyclonal)	Beijing Biosynthesis Biotechnology CO.
AMPK alpha-1	AMP activated kinase	bs-1115R	60kd	Rabbit (polyclonal)	Beijing Biosynthesis Biotechnology CO.
Phospho-AMPK alpha-1 (Thr172)	Phophorylated AMPK	bs-4002R	60kd	Rabbit (polyclonal)	Beijing Biosynthesis Biotechnology CO.
TAK1	Transforming growth factor (TGF_{β}) activated kinase-1	bs-3585R	67kd	Rabbit (polyclonal)	Beijing Biosynthesis Biotechnology CO.
CaMKKa	Ca^{2+}/calmodulin-dependent protein kinase alpha	bs-11247R	56kd	Rabbit (polyclonal)	Beijing Biosynthesis Biotechnology CO.
CaMKKb	Ca^{2+}/calmodulin-dependent protein kinase beta	bs-6253R	55kd	Rabbit (polyclonal)	Beijing Biosynthesis Biotechnology CO.
LKB1	LKB1	bs-3948R	48kd	Rabbit (polyclonal)	Beijing Biosynthesis Biotechnology CO.
ATPase5a	ATP synthetase5a	bs-2435R	13kd	Rabbit (polyclonal)	Beijing Biosynthesis Biotechnology CO.
ATPase5b	ATP synthetase5b	bs-8600R	51kd	Rabbit (polyclonal)	Beijing Biosynthesis Biotechnology CO.

anti-rabbit or donkey anti-goat Ab respectively for 1 h at RT. After three washes, the blots were imaged.

2.7. Immunofluorescent staining (IHF)

Testis sections (5 μm) were prepared and subjected to antigen retrieval and immunostaining as previously described (Liu et al., 2016). Briefly, sections were first blocked with normal goat serum in PBS, followed by incubation with primary Abs (1:100 in PBS-0.5% Triton X-100; Bioss Co. Ltd. Beijing, PR China) at 4 °C overnight. The primary Abs included rabbit anti-connexin 43 and rabbit anti-occludin (Table 1). After a brief wash, sections were incubated with an Alexa 546-labeled goat anti-rabbit secondary Ab (1:100 in PBS; Molecular Probes, Eugene, OR) at RT for 30 min and then counterstained with 4′,6-diamidino-2-phenylindole (DAPI). The stained sections were examined using a Leica Laser Scanning Confocal Microscope (LEICA TCS SP5 II, Germany). Three animal samples from each of the control- and cysteamine-treated groups were analyzed.

2.8. Statistical analyses

The data were statistically analyzed by SPSS statistical software (IBM Co., NY) using ANOVA. Comparisons between groups were tested by One-Way ANOVA analysis and the LSD test. All groups were compared with each other for every parameter (mean ± SEM). Differences were considered significant at $p < 0.05$.

3. Results

3.1. No effect on sheep body weight gain

Two doses (10 and 20 mg/kg BW/day) of cysteamine were used in this investigation in order to identify dose response effects and these two doses were relatively low because it is known that high doses cause gastrointestinal (GI) problems. The objective of this investigation was to explore the effects of cysteamine on spermatogenesis and the underlying mechanisms. Cysteamine did not affect BW gain (Fig. 1A). At the beginning of the study (age: 2.5 months), average BW was similar for the control and cysteamine treatment groups. After 6 months of treatment (age: 8.5 months), the average BW was still similar for all groups even though it was a little lower in the 10 mg/kg BW treatment group (not significant). Average BW gain was also similar for all three groups.

3.2. Decrease in spermatozoa quality

After 5.5 months of treatment (age: 8 months), semen samples were collected and spermatozoa motility and concentration were assessed using CASA. Firstly, it was found that the 20 mg/kg BW cysteamine treatment significantly decreased spermatozoa concentration, while 10 mg/kg did not (Fig. 1B). The 10 mg/kg treatment significantly increased spermatozoa motility through an increase in grade A and B spermatozoa; however, 20 mg/kg significantly decreased spermatozoa motility through an increase in grade C and D spermatozoa (immotile) and a decrease in grade A and B spermatozoa (Fig. 1C) compared with the control group.

3.3. Increase in blood lymphocytes

After 6 months of treatment, whole blood samples were collected and routine blood tests were performed. The treatment level of 20 mg/kg did not change the total number of white blood cells; however, it significantly increased the lymphocytes by 7.0 ± 1.2% compared to control samples. The treatment of 10 mg/kg did not affect blood lymphocytes.

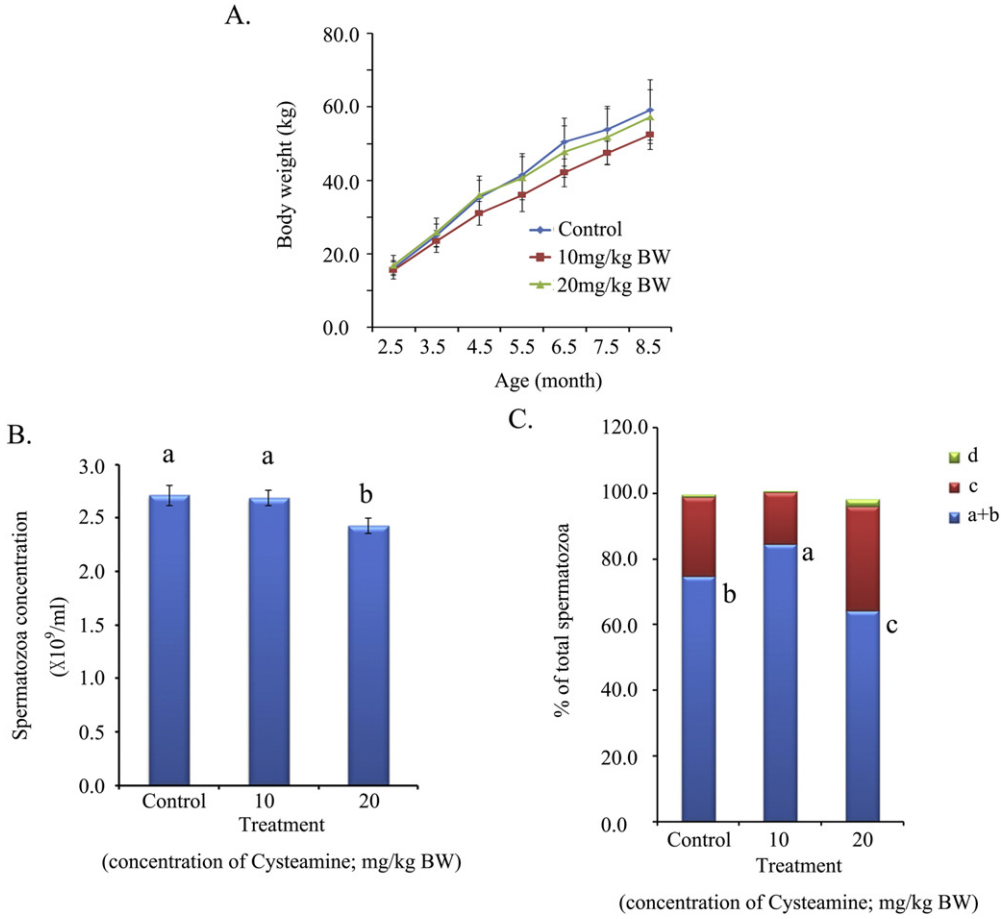

Fig. 1. Effects of cysteamine on sheep body weight and spermatogenesis. A. Sheep body weight. X-axis is the treatment time (months), and Y-axis is the body weight (kg). B. Spermatozoa concentration by CASA analysis. Y-axis = number of spermatozoa ($\times 10^9$/mL), X-axis = the treatment concentration (mg/kg BW). C. Spermatozoa motility determined by CASA analysis. Y-axis = % of total cells, X axis = the treatment concentration (mg/kg BW).

3.4. Increase in blood aspartate aminotransferase (ALT)

ALT and AST are two important indicators of liver damage. The 20 mg/kg BW cysteamine treatment slightly increased the activity of ALT but not AST in sheep blood (Fig. 2A and B), suggesting that this level might cause slight liver problems. T-SOD is a redox enzyme; cysteamine had no effect on this enzyme, which indicated that neither treatment affected the redox system in sheep (Fig. 2C).

3.5. Alteration in the levels of testosterone and estrogen

Hormones, especially FSH, LH, T, and E control spermatogenesis, and their levels were determined in the current study. The 10 mg/kg BW cysteamine treatment significantly increased levels of T and E, but not FSH or LH (Fig. 3A, B, C, and D). The 20 mg/kg treatment slightly decreased E (Fig. 3D; $p = 0.08$); however, this treatment had no effect on the other three hormones. Growth hormone also plays a role in spermatogenesis; however, neither treatment produced an effect in this case (Fig. 3E).

Since 10 mg/kg BW cysteamine increased plasma T and E levels, the protein levels of steroid hormone production enzymes were investigated by Western blotting (WB). Seven proteins: StAR, CYP11A1, CYP17A1, Aromatase, HSD17b1, HSD17B4, and HSD3B1 were analyzed. After 6 months of treatment, it was found that the protein levels of StAR, CYP17A1, and HSD17b4 were elevated by the 10 mg/kg treatment; however, these three proteins were decreased by the 20 mg/kg treatment (Fig. 4A). However, CYP11A1, Aromatase, HSD17b1, and HSD3b1 remained unchanged. The data matched well with plasma T and E levels. This indicated that cysteamine might act as an endocrine disruptor.

3.6. Reduction in junction protein levels in the testes

H&E staining (histopathology) indicated that the 10 or 20 mg/kg BW cysteamine treatment did not alter the structure of testis tubules (data not shown). However, Western blotting revealed that the junction protein levels of connexin 43 and occludin were decreased by the 20 mg/kg treatment (Fig. 4B). The data were also confirmed by IHF (Fig. 5A and B). This suggested that the 20 mg/kg treatment may affect the dynamics of the blood-testis barrier. Claudin 11 remained unchanged by both the 10 and 20 mg/kg treatments.

3.7. Unbalance in energy levels in sheep testis and spermatozoa

Because energy also plays an import role in spermatogenesis, the protein levels of ATP production enzymes APTase 5A and ATPase 5B were analyzed using Western blotting. The 20 mg/kg BW cysteamine treatment decreased the protein level of ATPase 5A, but not ATPase 5B (Fig. 6A). Furthermore, AMPK, a sensor of cell energy, was also detected in the testes. p-AMPK was increased by the 20 mg/kg treatment, which further suggested that energy was decreased by this treatment (Fig. 6A). AMPK itself remained unchanged. p-AMPK can be activated by the CaMKK1, CaMKK2, TAK1, or LKB1 pathways. LKB1 was decreased by the 20 mg/kg treatment (Fig. 6A), but CaMKK1, CaMKK2, and TAK1 remained unchanged. In spermatozoa, AMPK, p-AMPK, and LKB1 were

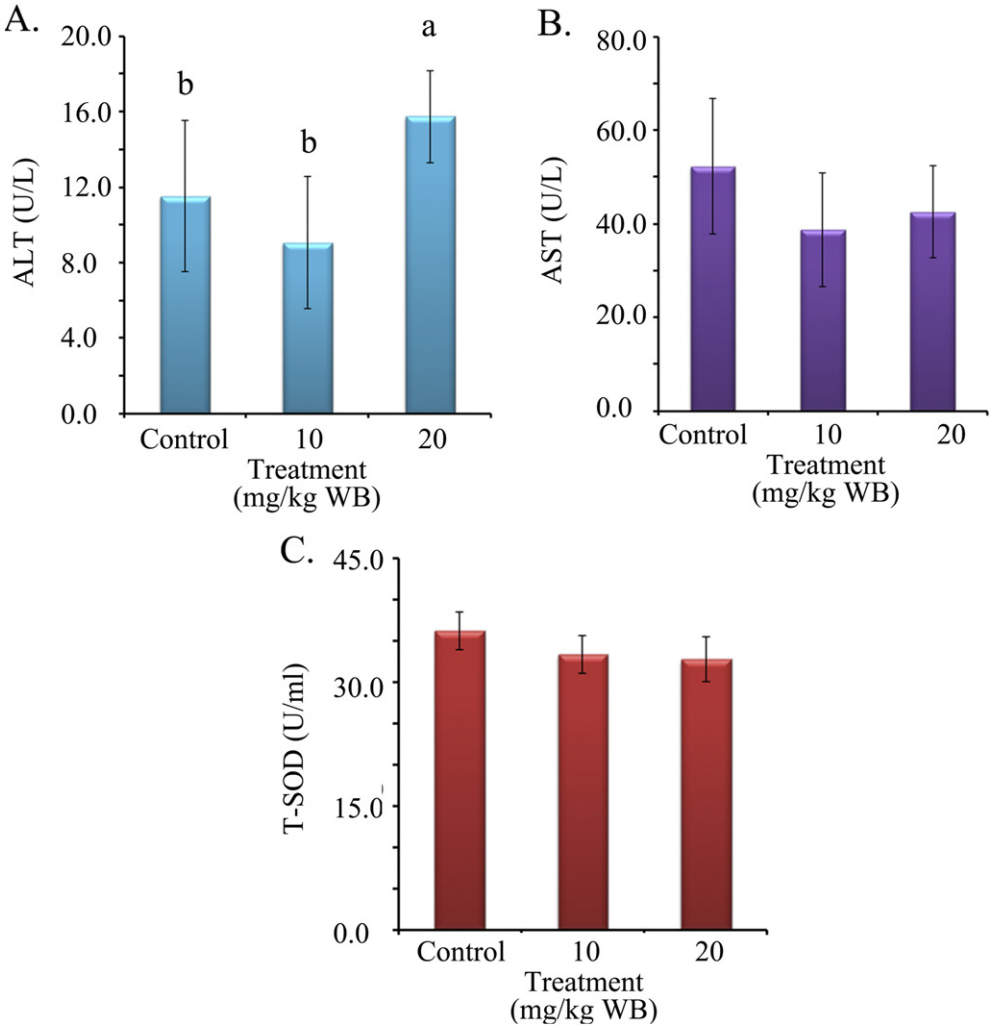

Fig. 2. Plasma ALT, AST and T-SOD levels. A. Plasma ALT levels, X-axis is the treatment time (months), and Y-axis is the ALT level (U/L). B. Plasma AST levels, X-axis is the treatment time (months), and Y-axis is the AST level (U/L). C. Plasma T-SOD levels, X-axis is the treatment time (months), and Y-axis is the T-SOD level (U/mL).

also elevated by the 20 mg/kg treatment, but not by 10 mg/kg, which indicated that the 20 mg/kg treatment also disrupted energy balance in spermatozoa (Fig. 6B).

3.8. Reduction in PIWIL1 and STK31 in sheep testis and/or spermatozoa

PIWIL1 and STK31 play an important role in both spermatogenesis and spermatozoa motility. The 20 mg/kg treatment caused a reduction in these two protein levels in sheep testis (Fig. 6C). Furthermore, spermatozoa STK31 was decreased by the 20 mg/kg treatment (Fig. 6D).

3.9. Disruption in redox balance and apoptosis pathway in spermatozoa

Catalase, SOD, and GPX are the three important redox enzymes that play a vital role in maintaining the redox balance. It was found that both 10 and 20 mg/kg treatments decreased GPX1 protein levels in spermatozoa; however, levels of SOD were increased by both these treatments (Fig. 6E). Apoptosis markers Bcl-2, Bcl-xl, Bax, caspase 3, and caspase 8 were analyzed in the sheep spermatozoa samples. It was found that the 20 mg/kg treatment decreased Bcl-2 protein levels but did not affect the other proteins (Fig. 6E). The data was also confirmed by IHF (Fig. 7). Bcl-2 is an anti-apoptosis marker. This indicated that apoptosis may have been increased by the 20 mg/kg treatment in spermatozoa.

4. Discussion

Although cysteamine has been used for more than three decades for the treatment of cystinosis and it is a promising compound for many other applications, it has resulted in many adverse effects in animals and humans. It causes lesions on patient organs and it induces ulcerogenic problems and developmental or even embryonic development issues (Beckman et al., 1998; Besouw et al., 2011; Selye and Szabo, 1973). Recently, we found that cysteamine inhibited peripubertal sheep mammary gland development and decreased birth rate.

Spermatogenesis is a very complex process that is regulated by the hypothalamic-pituitary gonadal axis, the male reproductive organs, and endocrine and paracrine factors involved in the control of sperm production and the release of androgens (Schlatt and Ehmcke, 2014). As cysteamine has been mainly used for treating cystinosis in children, its developmental safety has raised many concerns. Besouw et al. report that cysteamine causes embryo malformations, intrauterine growth retardation, and fetal death. However, the effects of cysteamine on spermatogenesis have not as yet been reported. In the current investigation, it was found that cysteamine (10 or 20 mg/kg BW/day) did not affect sheep BW gain. The 20 mg/kg treatment decreased spermatozoa concentration and spermatozoa motility. On the other hand, the 10 mg/kg treatment did not affect spermatozoa concentration, but did increase spermatozoa motility. The 20 mg/kg level increased blood

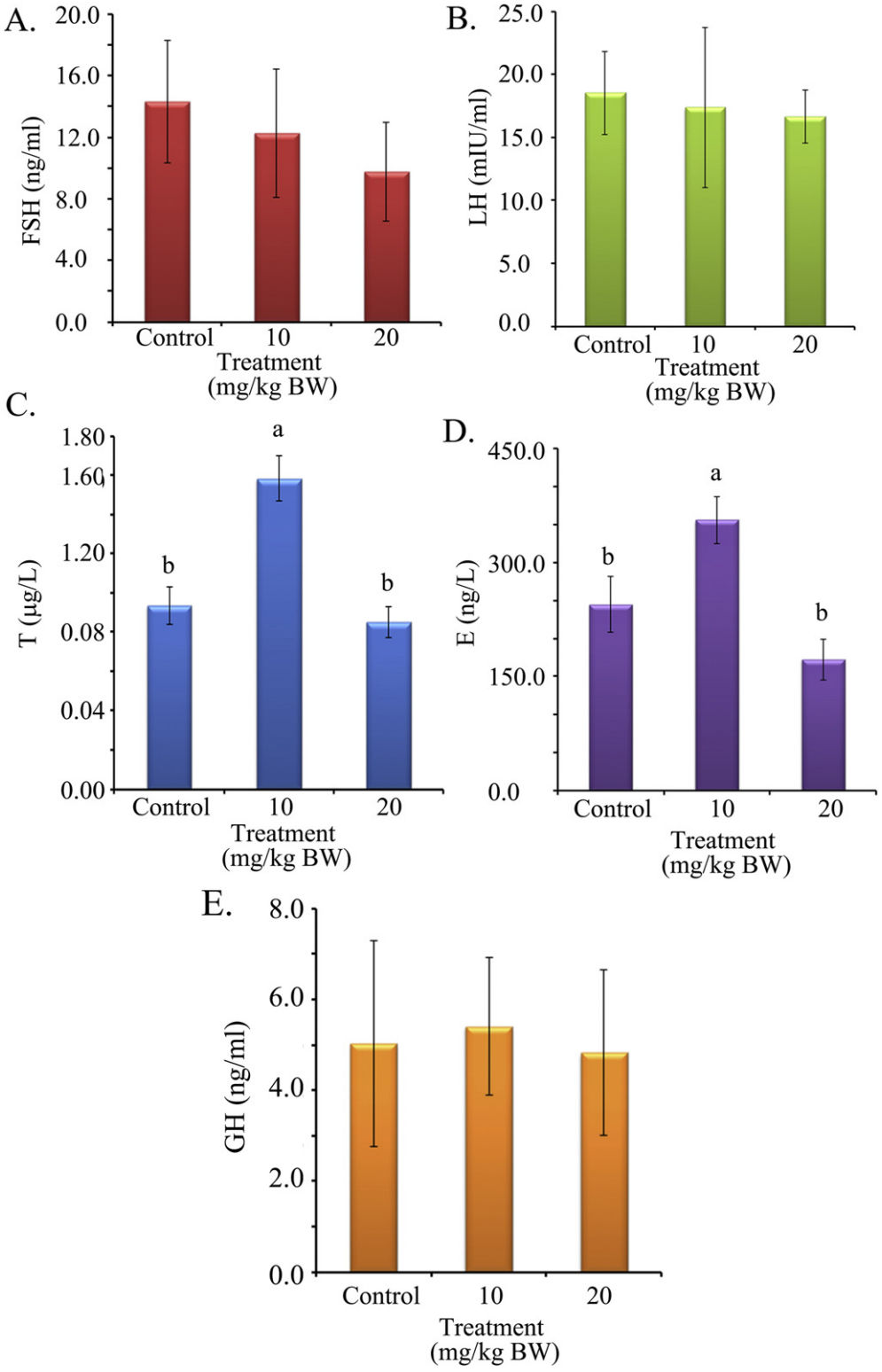

Fig. 3. Plasma hormone levels. A. Plasma FSH levels, x-axis is the treatment (mg/kg BW), and y-axis is the FSH level (ng/mL). B. Plasma LH levels, x-axis is the treatment (mg/kg BW), and y-axis is the LH level (mIU/mL). C. Plasma T levels, x-axis is the treatment (mg/kg BW), and y-axis is the T level (μg/L). D. Plasma E levels, X-axis is the treatment (mg/kg BW), and y-axis is the E level (ng/L). E. Plasma GH levels, X-axis is the treatment (mg/kg BW), and y-axis is the FSH level (ng/mL).

lymphocytes after 6 months of treatment which indicated that cysteamine might have some effect on immune response (Schönfeld et al., 1993). The 20 mg/kg treatment slightly increased plasma ALT level; however, the 10 mg/kg treatment had no effect on ALT or AST. This suggested that high levels of cysteamine might have a small effect on liver function. Furthermore, plasma growth hormone was not significantly changed by the 10 or 20 mg/kg treatments. This matched well with the BW data where cysteamine treatments had no effect.

It is well known that LH stimulates the Leydig cells of the testes to produce T and that this is a key event of spermatogenesis. FSH also regulates spermatogenesis (Chauvigné et al., 2012; Huhtaniemi, 2015). Furthermore, E plays a critical role in modulating libido, erectile

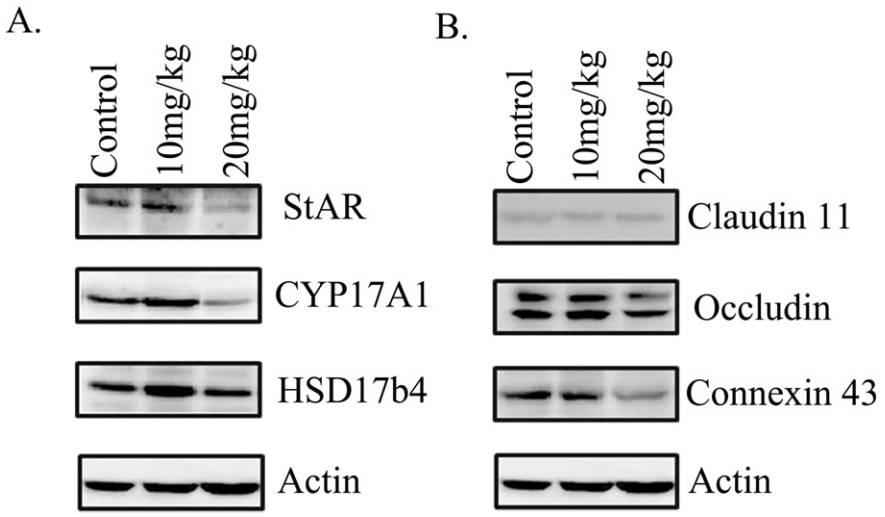

Fig. 4. Western blotting for steroid hormone production enzymes and junction proteins in sheep testis. A. 20 mg/kg cysteamine treatment decreased the protein levels of StAR, CYP17A1 and HSD17b4 in sheep testis. B. 20 mg/kg cysteamine treatment decreased the protein levels of occluding and connexin 43 in sheep testis.

function, and spermatogenesis during male sexual function (Schulster et al., 2016; Dumasia et al., 2016). In the current investigation, plasma FSH or LH concentration were not affected by cysteamine treatments. Plasma T and E levels were elevated by 10 mg/kg treatment while in 20 mg/kg treatment plasma E level was decreased even though the reduction was not significant. T and/or E production enzymes CYP17A1 and HSD17b4 were increased by the 10 mg/kg treatment. The 20 mg/kg treatment decreased T and E production enzymes StAR and CYP17A1. The data suggested that, through alteration in T or E production, cysteamine may regulate spermatogenesis. This is the first study to report that cysteamine regulated T or E production in males and that it may possibly be an endocrine disruptor.

Connexin 43, a constitutive protein of gap junctions, plays a critical role in the development of germ cells during fetal development and during spermatogenesis in adults (Kidder and Cyr, 2016). It interacts with the proteins of tight junctions (occludin and claudin), which are major components of the blood-testis barrier (Carette et al., 2013; Morrow et al., 2010; Pointis and Segretain, 2005). In the current investigation, it was found that the 20 mg/kg BW cysteamine treatment decreased the protein levels of connexin 43 and occludin; however, the 10 mg/kg treatment had no effect on these proteins. The data suggested that a higher concentration of cysteamine might break the blood-testis barrier to inhibit spermatogenesis. Piwil1 plays a central role in spermatogenesis, which ensures stable cell division rates in mammalian

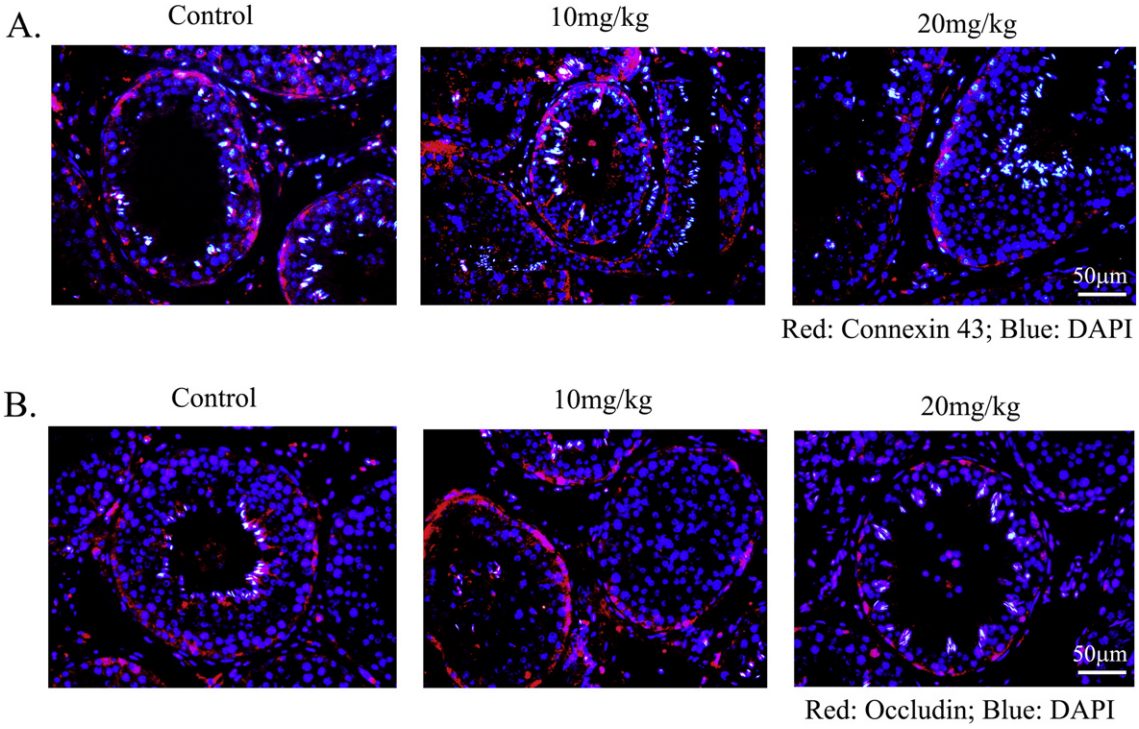

Fig. 5. IHF images for connexin 43 and occludin in sheep testis samples. A. 20 mg/kg cysteamine decreased the protein levels of connexin 43 in sheep testis. Red: Connexin 43 staining; Blue: DAPI staining for nucleus. B. 20 mg/kg cysteamine decreased the protein levels of occludin in sheep testis. Red: occludin staining; Blue: DAPI staining for nucleus. Scale bar: 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

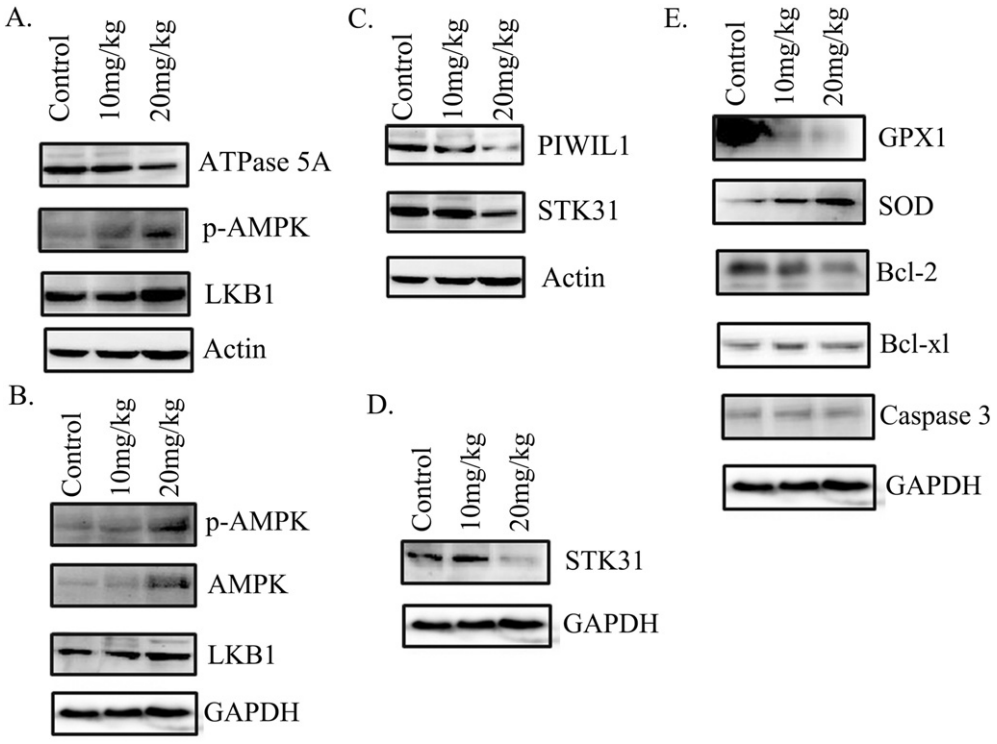

Fig. 6. Western blotting for proteins related to energy status and apoptosis in sheep testis samples. A. 20 mg/kg cysteamine treatment decreased the protein of APTase 5A, and increased the protein levels of p-AMPK and LKB1 in sheep testis. B. 20 mg/kg cysteamine treatment increased the protein levels of p-AMPK, AMPK and LKB1in sheep spermatozoa. C. 20 mg/kg cysteamine treatment decreased the protein of PIWIL1 and STK31 in sheep testis. D. 20 mg/kg cysteamine treatment decreased the protein of STK31 in sheep spermatozoa. E. 20 mg/kg cysteamine treatment decreased the protein of GPX1 and Bcl-2, and increased the protein level of SOD in sheep spermatozoa.

germline cells (Xu et al., 2016). STK31, a member of the Tudor-domain-containing proteins (TDRDs) subfamily (also termed as TDRD8), is expressed throughout spermatogenesis during postnatal development where it plays a vital role. It also retains its localization to the equatorial segment of the acrosome during epididymal maturation, capacitation, and the acrosome reaction (Bao et al., 2012). In the current investigation, it was found that the 20 mg/kg treatment decreased PIWIL1 and STK31 protein levels in testis, and it also reduced the protein level of STK31 in spermatozoa, which further indicated higher concentration of cysteamine might block the process of spermatogenesis.

Cellular energy status is also critical for spermatogenesis (Miki, 2007; Garrett et al., 2008). The energy sensor, AMPK, is a vital regulator of energy balance (Hardie, 2011; Carling et al., 2012), i.e. the maintenance of energy levels under ATP-limiting conditions (Hardie, 2011). In the current investigation, it was found that p-AMPK was increased in both testicular tissue and in spermatozoa. Furthermore, LKB1, one activator of p-AMPK, was elevated. This indicated that cysteamine might decrease cellular energy levels, which matched well with the finding that APTase 5A was decreased by the 20 mg/kg treatment. Moreover, it suggested that cysteamine might activate LKB1, which subsequently

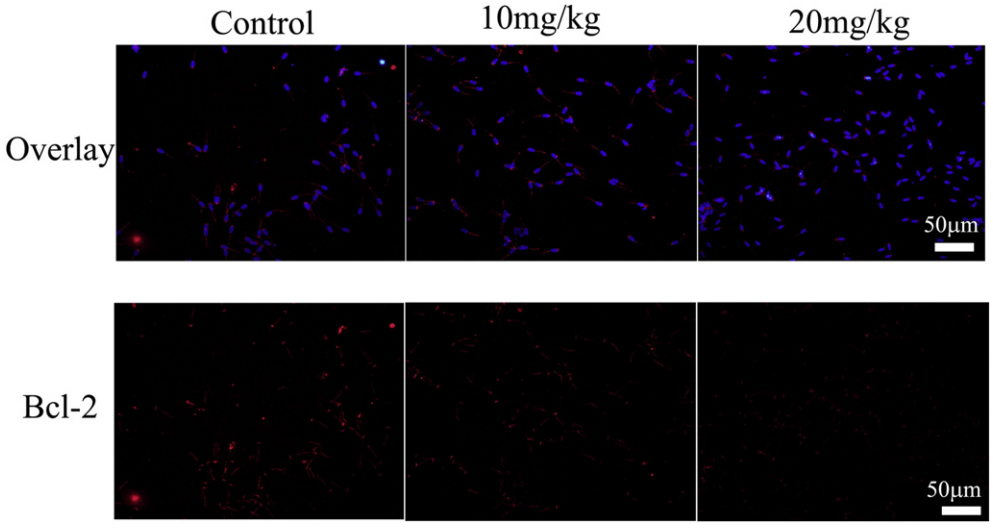

Fig. 7. IHF images for Bcl-2 in sheep spermatozoa. 20 mg/kg cysteamine decreased the protein levels of Bcl-2 in sheep spermatozoa. Red: Bcl-2 staining; Blue: DAPI staining for nucleus. Scale bar: 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

activates p-AMPK. Mitochondrial membrane potential is important for spermatozoa motility (Hurtado de Llera et al., 2015). The mitochondrial membrane potential and percentage of apoptosis cells were also determined in this study however cysteamine affected neither the spermatozoa mitochondrial membrane potential nor the percentage of apoptosis cells. As we found that 10 mg/kg and 20 mg/kg cysteamine treatments did not alter the sheep body weight gain and just 20 mg/kg treatment caused a little problem on sheep liver which suggested that the doses of cysteamine used in this investigation were moderate. Among apoptosis related proteins analyzed in this study, just Bcl-2 was found to be decreased by 20 mg/kg cysteamine treatment which indicated that Bcl-2 might be the sensitive factor to be affected by 20 mg/kg cysteamine treatment.

Besouw et al. (2010) suggested that cysteamine might negatively affect the fertility in male cystinosis patients with inhibition in spermatogenesis. Our data supported their suggestion. And cysteamine affected spermatogenesis in a dose-dependent manner. Low dose (10 mg/kg BW) treatment increased spermatozoa motility while a higher dose of 20 mg/kg decreased spermatozoa concentration and motility. Therefore, cysteamine is suggested to use at low dose as much as possible.

In conclusion, the effects of cysteamine on spermatogenesis were dose dependent. Low dose (10 mg/kg BW) cysteamine treatment increased spermatozoa motility. However, a higher dose of 20 mg/kg decreased ovine spermatozoa concentration and motility. The decrease in spermatozoa concentration and motility might be due to a reduction in testicular steroid hormone production, a reduction in energy in the testis and spermatozoa, disruption in the blood-testis barrier, or breakdown in the vital signaling pathways involved in spermatogenesis. The inhibitory effects of cysteamine on sheep spermatogenesis may be useful to model its effects on young male patients with cystinosis or other diseases who are treated with cysteamine. Further studies on spermatogenesis that focus on patients treated with cysteamine during the peripubertal stages are warranted.

Conflict of interest

None of the authors have any conflict of interest to declare.

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Transparency document

The Transparency document associated with this article can be found, in online version.

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