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High salt diet decreases reproductive performance in rams and down-regulates gene expression of some components of the renin-angiotensin system in the testis

Yi Fang [a, b], Rongzhen Zhong [a], Xuezhao Sun [c], Daowei Zhou [a, *]

[a] Jilin Provincial Key Laboratory of Grassland Farming, Northeast Institute of Geography and Agoecology, Chinese Academy of Sciences, Changchun, Jilin 130062, China
[b] Laboratory Animal Center, Jilin University, Changchun, Jilin 130062, China
[c] Jilin Agricultural Science and Technology University, Jilin, Jilin 132011, China

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ABSTRACT

Although salt-tolerant plants can be used to combat dryland salinity, these plants contain high concentrations of salt (NaCl), which may have deleterious effects if fed to livestock. Twenty-four Merino rams (9 mo of age) with similar body weight were equally allocated to two groups and fed a normal- or high-salt diet (0.5 and 12% NaCl, respectively) for 3 mo. Rams fed the high-salt diet had lower live weight gains, higher water intake, smaller testes (466 ± 48.4 *vs* 604 ± 51.1 g) and reductions ($P < 0.05$) in spermatogenesis, ejaculate volumes (0.89 ± 0.18 *vs* 1.24 ± 0.15 mL/ejaculate), sperm concentration (14.3 ± 2.05 *vs* 22.3 ± 2.33 × 10^9 sperm/mL) and DNA integrity (DNA fragmentation rate: 5.85 ± 1.09 *vs* 1.13 ± 0.14%) compared to controls. For *in vitro* fertilization, although cleavage percentage was not significantly affected, hatching rate was lower (30.8 ± 3.81 *vs* 52.8 ± 4.08%, $P < 0.05$) for sperm from rams on the high-salt diet. Furthermore, the 12% salt diet decreased plasma concentrations of metabolic (leptin and insulin) and sex (T, FSH and LH) hormones, but did not affect the plasma renin-angiotensin system (RAS) component (REN, Ang II, ACE and ALD). Regarding components of the testicular RAS, the 12% salt diet decreased ($P < 0.05$) expression of REN, Ang II and AT2 mRNA, although ACE and AT1 were unaffected. Furthermore, the 12% salt diet decreased ($P < 0.05$) mRNA expression of key genes for spermatogenesis (Hsp70, c-kit and Cyclin A), and sex hormone receptors (AR, FSHR, LHR, CYP11A1 and CYP17A1), but there were no significant effects on key enzymes (LDH, SDH, AKP and ATPase) in the testis. In conclusion, the high-salt diet reduced ram reproductive performance; we inferred that changes in testis RAS may have had an important role in these reproductive defects.

1. Introduction

A major limitation of saline pasture systems is the excessive salt (NaCl) content of forage and drinking water for grazing animals. Typical plant communities in the west grassland of Jilin province of China, including *Leymus chinensi, Suaeda glauca, Chloris,* and *Puccinellia tenuiflora*, accumulate salt [1]. For example, salt content of *Suaeda glauca* is up to 317–331 g/kg DM [2], whereas several other forages have 15–41 g/kg DM, much greater than a salt content of 1.2–1.8 g/kg DM in most forage [3]. Furthermore, in saline areas, surface water contains up to 0.9–1.7% salt, exacerbating increased salt intake [4].

Ingestion of halophytic forages may have deleterious effects on growth [5], carcass grade [6], fetal wellbeing [4,7] and also reproduction of sheep, directly or indirectly, although mechanisms are not fully understood, especially in males. Most research has focused on ewes, with effects of high salt intakes (NaCl contents of 0.5–2% and 5–20% in water and diet, respectively), in non-breeding ewes [8] and during the breeding season [9], pregnancy and lactation [10]. There is evidence that high salt intakes reduce reproductive performance, with greater sensitivity to high salt from drinking water compared to diet, in males compared to females, and in young ewes versus adults. In males, there are limited data regarding effects of high-salt intakes on reproduction. For example,

* Corresponding author. Northeast Institute of Geography and Agroecology, Chinese Academy of Sciences, Changchun, Jilin 130012, China.
E-mail address: zhoudaowei@iga.ac.cn (D. Zhou).

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in mice, high-salt diets (3.5 and 5% NaCl diet for 6–8 wk) reduced testis mass and spermatogenesis [11], with effects probably regulated by the renin-angiotensin system (RAS) [12].

The RAS is a sodium ion direct-sensing system [13]. In addition to the kidney-liver-cardiovascular system, testis, epididymis and prostate are rich in RAS components and RAS activity [14]. Renin (REN) acts on angiotensinogen to form angiotensin (Ang) I, which is converted by angiotensin converting enzyme (ACE) to Ang II in various organs. Shortly before puberty, REN, ACE and Ang II in the testis increase concomitant with plasma gonadotropin concentrations, with Ang II concentrations 3–5 times higher in seminal plasma versus serum [15], suggesting that these components are important for puberty, and can affect male reproductive capacity, including sperm maturation and release [14–18]. Specialized mechanisms have evolved to modulate testis-specific gene expressions and promote transition from primordial germ cells to spermatogonial stem cells and finally to mature sperm [18].

Our objective was to determine effects of a high-salt diet on reproductive activity in rams. We tested the hypothesis that a high-salt decreases sperm quality and testis activity, and alters plasma hormone concentrations, testis RAS and specific gene expressions.

2. Materials and methods

All procedures involving animals were reviewed and approved by the Chinese Academy of Science Animal Care and Use Committee. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise indicated.

2.1. Experimental design and treatments

Twenty-four Merino breeding rams, 9 mo old, with uniform body weight (61.53 ± 3.43 kg) and body condition score (3.6 ± 0.1 units, scale 0–5 [19]), were used. All rams were treated with anthelmintics (Weiyuan, Hebei, China) 2 mo before the beginning of the experiment. The experiment was conducted during the breeding season (August–October, i.e. late summer to late autumn) at the Changling Grassland Farming Ecological Experimental Station of Chinese Academy (44°33′ N, 123°31′ E).

2.2. Experimental diets and feeding

Two pelleted diets containing alfalfa, corn stalk, corn and soybean meal were obtained from a commercial manufacturer (Jinweinong, Jiangsu, China). The treatment diet was formulated to contain 12% NaCl to mimic the salt concentration in a 30% halophytic forages-based diet, whereas the control diet contained 0.5% NaCl. There were 12 rams in each group. For the 2-wk acclimation period, rams were confined to individual metabolic crates in a controlled temperature (20–23 °C) room with a 12:12 h light-dark cycle, and relative humidity that ranged from 40 to 90%.

During the 3-mo experimental period, all rams were fed at 06:00–08:00 and 16:00–18:00, and amounts of feed offered and refused was recorded. Rams in the high-salt group were fed *ad libitum*; if no residual feed was present, the amount fed the following day was increased by 100 g. Intake of the 0.5% salt group was paired with individuals in the 12% salt group based on body weight. Fresh water was available twice daily over the experimental period; water consumption was not controlled and was determined on a group basis by fitting a flow meter to the water line supplying each small holding paddock.

2.3. Body weight and blood sampling

Every 10 d, body weight was determined and blood samples were collected by jugular venipuncture using heparinized vacutainers (Beyotime, Jiangsu, China). To minimize effects of circadian rhythms on hormone measurements, sample collection was consistently done between 09:00 and 10:00 in resting conditions, always by the same operator. Blood was placed into chilled tubes (Beyotime) containing 0.125 M EDTA and 0.025 M o-phenanthroline, centrifuged (1000 × g at 4 °C for 15 min), and plasma was immediately extracted and stored at −20 °C.

Commercial enzyme-linked immunosorbent assays (ELISA, DRG Diagnostics, Marburg, Germany), performed according to manufacturers' instructions, were used to determine plasma concentrations of the following hormones (with assay sensitivity included after the description of the assay): Leptin (EIA 2395, 0.7 ng/mL), insulin (HYE 5361, 1.61 μIU/mL), REN (EIA 5125, 0.81 pg/mL), Ang II (EIA 5537, 0.95 pg/mL), ACE (EIA 5661, 1.8 pg/mL), aldosterone (ALD, EIA 5298, 5.7 pg/mL), testosterone (T, HYE 5376, 0.139 ng/mL), follicle stimulating hormone (FSH, EIA 1288, 0.856 mIU/mL) and luteinizing hormone (LH, HYE 5362, 0.9 mIU/mL). Intra- and inter-assay coefficients of variations were 2.0–12.2 and 3.7–14.9%, respectively.

2.4. Semen collection

Semen was collected by electro-ejaculation (BTS-DC, Beijing, China) every 3 d from 75 to 90 d of the experimental period. On each collection day, rams were collected twice; first ejaculates were discarded and a total of 120 s ejaculates were examined.

2.5. Sperm viability

Eosin-nigrosin stain was prepared by dissolving 1.40% eosin-Y and 8.72% nigrosin (both from Beyotime), and 2.53% sodium citrate in distilled water. Sperm smears were prepared by mixing a drop of semen (1.0×10^6 sperm/mL) with two drops of stain on a warm slide and spreading the mixture with a second slide. Sperm with only partial or complete purple staining were considered nonviable; only sperm with strict exclusion of stain were considered viable.

2.6. Acrosome integrity

To assess sperm acrosomal integrity, 100 μL of a 1.0×10^6/mL sperm suspension was fixed in 500 μL of 1% formal citrate (2.79% tri-sodium citrate dehydrate and 0.37% formaldehyde in distilled water). Acrosomes of 200 sperm were examined under 1000 x magnification using a phase contrast microscope (Olympus BX60, Olympus, Japan). Acrosome integrity was defined as the presence of a normal apical ridge [20].

2.7. Hypo-osmotic swelling test (HOST)

In brief, 50 μL of a 1.0×10^6/mL sperm suspension was incubated with 300 μL of hypo-osmotic solution (2.04% fructose and 0.71% sodium citrate in distilled water) at 37 °C for 60 min. Then, a 100 μL drop of the mixture was spread on a warm slide with a cover slip and 200 sperm were evaluated under 1000 x magnification using a phase contrast microscope (Olympus BX60). The morphology of sperm (normal tails versus swollen or coiled tails) was recorded [21].

2.8. Evaluation of DNA fragmentation

Sperm DNA fragmentation was evaluated by TUNEL [22]. Briefly, 1.0×10^6/mL samples were fixed in 4% paraformaldehyde (PFA, 500 μL, 4% in PBS, pH 7.4) (Beyotime) and processed for TUNEL