RESEARCH ARTICLE

Reproductive responses of the male Brandt's vole, *Lasiopodomys brandtii* (Rodentia: Cricetidae) to tannic acid

Xin Dai[1], Ling-Yu Zhou[1], Ting-Ting Xu[1], Qiu-Yue Wang[1], Bin Luo[1], Yan-Yu Li[1], Chen Gu[1], Shi-Ping Li[1], Ai-Qin Wang[1], Wan-Hong Wei[1], Sheng-Mei Yang[1]

[1]College of Bioscience and Biotechnology, Yangzhou University. 48 East Wenhui Road, Yangzhou 225009, China.
Corresponding author. Sheng-Mei Yang (smyang@yzu.edu.cn)

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ABSTRACT. Tannins are polyphenols that are present in various plants, and potentially contain antioxidant properties that promote reproduction in animals. This study investigated how tannic acid (TA) affects the reproductive parameters of male Brandt's voles, *Lasiopodomys brandtii* (Radde, 1861). Specifically, the anti-oxidative level of serum, autophagy in the testis, and reproductive physiology were assessed in males treated with TA from the pubertal stage. Compared to the control, low dose TA enhanced relative testis and epididymis weight and sperm concentration in the epididymis, and significantly increased the level of serum superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px). mRNA levels of autophagy related genes LC3 and Beclin1 decreased significantly with low dose TA compared to the control. However, compared to the control, high dose TA sharply reduced the levels of serum SOD, GSH-Px, CAT, serum testosterone (T), and mRNA level in steroidogenic acute regulatory protein (StAR) in the testis. Both sperm abnormality and mortality increased with high dose TA compared to the control and low dose TA. Collectively, this study demonstrated that TA treatment during puberty had a dose-dependent effect on the reproductive responses of male Brandt's voles. TA might mediate autophagy in the testis, through both indirect and direct processes. TA mainly affected the reproductive function of male Brandt's voles by regulating anti-oxidative levels. This study advances our understanding of the mechanisms by which tannins influence reproduction in herbivores.

KEY WORDS. Antioxidative, autophagy, puberty, reproduction.

INTRODUCTION

Tannins are a group of naturally occurring polyphenols that are widespread in the plant kingdom (Swain 1979), and are considered to be plant secondary metabolites (PSMs) because they directly contribute to plant metabolic processes (Pepi et al. 2009). The phenolic hydroxyl groups of tannins bind and inhibit the digestive enzymes of animals, inducing the formation of complexes with plant proteins, as well as with metal ions, amino acids, and polysaccharides. Because these complexes are not readily degraded by the digestive enzymes of mammals, tannins might serve as anti-nutritional factors in many animals (Chung et al. 1998), adversely affecting the digestibility and nutrient absorption of food (Hagerman and Klucher 1986, Mehansho et al. 1987). However, tannins also have beneficial biological effects. For example, tannins might act as antioxidants (Wu et al. 2004, Andrade et al. 2005, Türk et al. 2008, Gülçin et al. 2010, Bouki et al. 2013). Tannins also

promote reproduction in animals (Ramírez-Restrepo and Barry 2005, Ramírez-Restrepo et al. 2005, Yousef 2005, Yakubu et al. 2008, Türk et al. 2008). Some researchers have speculated that tannins might benefit the reproduction of animals through their antioxidative properties (Türk et al. 2008). However, this correlation and its mechanism require elucidating. Within the antioxidant system, superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) are the enzymes that play predominant roles in countering the adverse effects of oxidative stress (Fridovich 1978, Imre et al. 1984, Hazelton and Lang 1985), and are also considered to affect sperm function as a suppressor or scavenger of free radicals (Sikka 1996, Vernet et al. 2004).

Autophagy (or programmed cell death type 2), is an evolutionarily conserved mechanism involved in the degradation and recycling of misfolded proteins and excess or dysfunctional subcellular organelles (Galluzzi et al. 2007). Beclin1 is the mammalian autophagy gene, and is important for the localization of autophagic proteins to a pre-autophagosomal structure, in

addition to being a critical regulator of autophagosome formation (Kang et al. 2011). LC3 (microtubule-associated protein 1 light chain 3) is a subfamily of essential proteins that contributes to multiple cellular processes, such as autophagy (Shpilka et al. 2011). In mammalian cells, LC3A is one isoform of LC3 that is rapidly cleaved to yield the cytosolic form, LC3-I (Yoshimori 2004, Li et al. 2011). After the induction of autophagy, LC3-I is covalently conjugated to phosphatidylethanolamine to form LC3-II (Ishibashi et al. 2011). Then, LC3-II binds tightly to pre-autophagosomal, autophagosomal and autolysosomal membranes to form a suitable marker for the autophagy process (Giatromanolaki et al. 2010). Autophagy is also important in the testes, and has been associated with spermatogenesis, sperm quality, and the secretion of testosterone (Zhang et al. 2012, Wang et al. 2014, Herpin et al. 2015). Several studies have reported the importance of autophagy in male reproductive dysfunction (Lim and Song 2014, Ma et al. 2015). Oxidative stress mediates autophagy (Slater et al. 1995, Lee et al. 2012), allowing it to scavenge those oxidized proteins and damaged mitochondria in organs (Hariharan et al. 2011, Malaviya et al. 2014). Because tannins have antioxidant properties, we hypothesized that tannins could mediate autophagy in animals.

The Brandt's vole, *Lasiopodomys brandtii* (Radde, 1861) is abundant in the grasslands of Inner Mongolia, China. It is a small, gregarious and, mainly, polygynous, seasonally reproductive mammal (from March to September) (Xie et al. 1994, Shi et al. 1999, Wan et al. 2002). It feeds on a wide variety of herbaceous monocotyledons and dicotyledons (Wang et al. 1992). Interestingly, plants favored by Brandt's voles universally contain tannins (Dai et al. 2014, Yin et al. 2017). Furthermore, few male Brandt's voles participate in reproduction during the breeding season in which they were born (Ren et al. 2016). The mechanism driving this phenomenon remains unclear. In parallel, knowledge about how tannins affect autophagy in Brandt's voles is not known. Thus, Brandt's vole is an ideal model for studying how tannins affect reproduction and autophagy in mammalian herbivores.

Puberty is a key period in the reproductive development of mammals, with individuals being more susceptible to external factors. Here, we investigated the reproductive responses of male Brandt's voles treated with different doses of tannic acid (TA) from puberty, with respect to oxidative stress and autophagy, and also examined the dose effects of TA on multiple parameters of these voles. Specifically, we evaluated the body weight, relative testis and epididymis weights, the concentrations of serum reproductive hormones, the activity of serum antioxidative enzymes (SOD, CAT, and GSH-Px), the quality of sperm, and the mRNA levels of autophagy-related genes (Beclin1 and LC3A) and steroidogenic acute regulatory protein (StAR), which encode key enzymes for testosterone synthesis (Stocco 2001, Payne and Hales 2004) in the testis, of Brandt's voles treated with TA from four weeks (pubertal stage) to 8 weeks (adult stage) of age, the latter of which is considered the age of sexual maturation in

Brandt's voles (Chen et al. 2017). The results are expected to advance our understanding on the mechanisms that cause tannins to influence reproduction in herbivores and the role of plant secondary metabolites on regulating herbivore populations.

MATERIAL AND METHODS

Our study was conducted during June-October 2018 at college of Bioscience and Biotechnology, Yangzhou University, China. Brandt's voles captured from the grasslands of Inner Mongolia were bred as the F0 generation in the animal group facility at Yangzhou University, Jiangsu Province, China. Environmental conditions were controlled at a temperature of 22 ± 1 °C, a relative humidity of 50 ± 5%, and a photoperiod of 12 hours light/12 hours dark (light period extending from 6:00 am to 6:00 pm). At 21 days of age, F1 generation male voles were weaned and housed separately in polypropylene cages, and were allowed to acclimate for another seven days until four weeks of age. Each pubertal vole was provided with 10 mL filtered tap water and rodent chow (containing 318.65 ± 29.81 µg/g tannin) ad libitum during this period. The nutrient contents of the rodent chow were as follows: crude protein, ≥18%; crude fat, ≥4%; crude fiber, ≥5%; ash, ≤8%; calcium, 1.0%–1.8%; and phosphorus, 0.6%–1.2%. Following acclimation, a cohort of 18 voles was randomly assigned to one of three groups (control, low dose, and high dose), with the same number of individuals in each group. The experiment lasted for four weeks.

A stock solution (6 mg/mL TA) was prepared by dissolving 3 g TA (Tianjin Kemiou Chemical Reagent Co., Ltd.) in 500 mL filtered tap water, and was stored at 4 °C. On the day of administration, the stock solution was brought to room temperature and diluted 2-fold with filtered tap water to obtain 3 mg/mL TA solution. The voles in each group received 10 mL filtered tap water (control group), 3 mg/mL TA solution (low dose group), and 6 mg/mL TA solution (high dose group), respectively, every two days. In the field, the average tannin contents per dry weight of the most preferred food plant species – *Leymus chinensis* (Trin.) Tzvel, 1968, *Setaria viridis* Beauv., 1817, and *Medicago sativa* Linn., 1753 – during the period from May to August are approximately between 3 mg/g and 7.5 mg/g (Yin et al. 2017). Naturally, pubertal voles probably consume at least 10 g of dry plant food each day (Wan et al. 2001), and thus, in the field, the daily tannin intake of pubertal voles is estimated to be between 30 and 75 mg. Accordingly, we established a high TA dosage (whereby voles received 30 mg TA per day via drinking water), such that voles received the lower limit of tannin consumed daily by pubertal voles in the field. Furthermore, the highest amount of tannin in the chow consumed by each vole was approximately 3.2 mg/day (in the laboratory, the amount of chow consumed daily by each pubertal vole was no more than 10 g), and thus, the total intake of tannin in the high-dose TA group was estimated to be 33.2 mg/day, which is still approximately equivalent to the lower limits of

natural tannin consumed by pubertal voles in the field. We thus believe that the applied dosage of TA was reasonable and approximately reflected the amount of tannin consumed daily by pubertal Brandt's voles under natural conditions. Low TA dosage (whereby voles received 15 mg TA per day via drinking water) was set to check the dose effects of TA. The day after administration, each vole received filtered tap water ad libitum. Throughout the experimental period, we ensured that 10 mL water or TA solution was consumed by Brandt's voles on each day of administration. Throughout the experiment, all voles were provided with standard rodent chow ad libitum.

Voles were weighed every week from four weeks to eight weeks in age. At eight weeks in age, all animals were weighed and decapitated after anesthetizing with ether. Blood samples were collected and kept at 4 °C overnight. Paired testes and epididymides were collected and weighed as soon as possible using a precision scale balance (± 0.001 g; ML203T/02, Mettler Toledo Co., Shanghai, China). Relative testis and epididymis weight were calculated as paired testes and epididymides weight (g) divided by body weight (g). After weighing, paired testes were immersed in RNA preservation liquid, and stored at –20 °C. The left epididymis was used to detect the concentration, mortality, and abnormality rate of sperm. Then, the serum was obtained by centrifugation at 3000×g for 30 minutes, and was stored at –80 °C. All procedures were approved by the Animal Care and Use Committee of the Faculty of Veterinary Medicine of Yangzhou University.

All procedures in our experiment were approved by the Animal Care and Use Committee of the Faculty of Veterinary Medicine of Yangzhou University.

The sequences we cloned have been submitted to GenBank as partial mRNA sequence for each gene (accession numbers for LC3A and Beclin1 were MK477699 and MK477700, respectively).

After weighing, the left epididymis isolated from each vole was immediately placed in 2 mL 0.01 M PBS, which had been warmed to 37°C. The caudal epididymis was cut open with eye scissors to release epididymal fluid. Then, the exuded epididymal fluid was incubated for three minutes at 37 °C. A 100 μL volume of diluted epididymal fluid was collected for staining using Typan Blue Staining Cell Assay Kit (Beyotime, China), according to manufacturer's instructions. A 10 μL solution was placed to a hemocytometer to determine sperm concentration and quantify the rate of sperm abnormality and mortality under a light micro-scope (20× objective). In total, 40 μL solution was checked for each epididymis. Sperm concentration was expressed in millions per mL. Dicephaly, double tails, short tail, microcephalic, and megacephalic sperm were defined as abnormal sperm based on Hao et al. (2009). A total of 200 sperms were observed per vole.

Serum hormones, were quantified in duplicate using an ELISA kit (LianShuo Biological Technology Co., Ltd., Shanghai, China), according to the manufacturer's instructions, and as previously reported (Dai et al. 2016). The hormones included luteinizing hormone (LH), follicle stimulating hormone (FSH) and testosterone (T), and serum anti-oxidative enzymes, namely SOD, CAT, and GSH-Px. The purity of all LH, FSH, T, SOD, CAT, and GSH-Px standard preparations was >95%. The intra- and inter-assay coefficients of variation were <9 and <15%, respectively, for all three hormones and three enzymes.

Total RNA was extracted and stored using the procedure established in our previous study (Dai et al. 2016). RNA samples of 1 μg were reverse-transcribed using the PrimeScript 1st strand cDNA synthesis kit (TaKaRa), following the manufacturer's instructions. Gene expression was measured by qPCR. The sequences of qPCR primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β-actin, and StAR were adopted from previous studies (Dai et al. 2016) (Table 1). Microtubule-associated protein 1 light chain 3 A (LC3A) and Beclin1 by using amino acid sequences from vertebrate animals corresponding to LC3A and Beclin1 that were retrieved from the National Center for Biotechnology Information (NCBI) database and aligned using Clustal X (Larkin et al. 2007). Two degenerate primer pairs suitable for cDNA synthesis were designed using j-CODEHOP from base-by-base (Rose et al. 1998, Rose et al. 2003, Boyce et al. 2009). The synthesized cDNA was cloned into pMD18-T vector, and sequenced by Sangon Biotech Company (Shanghai, China). The sequences were submitted to the GenBank (accession numbers for LC3A and Beclin1 were MK477699 and MK477700, respectively), and analyzed using the NCBI Primer Blast tool to design qPCR primers. The specificity of the primers was checked using PCR and the melt curve of qPCR, to ensure that no primer dimers or non-specific products were formed (Table 1). PCR reactions were conducted in a real-time PCR system (Applied Biosystems, Foster City, California, USA) using SYBR Premix EX Taq II (TaKaRa), as previously described (Dai et al. 2016). Standard curves were constructed for each gene via serial five-fold dilutions of cDNA. Amplification efficiency ranged between 0.9 and 1.0, confirming the validity of the comparative

Table 1. Primers used in the qPCR study.

	Forward (5'–3')	Reverse (5'–3')	Reference
LC3A	GCTTCGCCGACCGCTGTAA	ATCCGTCTTCATCCTTCTCCTG	Designed in the present study
Beclin1	GGTCGCTTGCCCAGTGTT	ACGGCAACTCCTTAGATT	
StAR	GGTCCTGCAAAAGATCGGGAA	GGCATCTCCCCAAAATGTGTG	Dai et al. 2016
β-actin	TTGTGCGTGACATCAAAGAG	ATGCCAGAAGATTCCATACC	
GAPDH	TGGCAAAGTGGAGATTGTTGCC	AAGATGGTGATGGGCTTCCCG	

quantification method. No amplification was detected in the absence of the template or non-RT control. The fold change of gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001), using β-actin (O'Shaughnessy et al. 2002) and GAPDH (Xi et al. 2011) as housekeeping genes for testis gene expression. The average ΔCt for the control group was used to calibrate each sample.

All variables were tested for normality and homogeneity by the Shapiro-Wilk and Levene test, and were transformed by log10, when necessary. The effect of TA doses on the body mass of voles was evaluated using repeated measures analysis, in which body weight on the fourth week was the covariate, followed by the least significant difference (LSD) post hoc test. The effect of TA on autophagy related gene expression in the testis, relative testis weight, expression of StAR in testis, serum hormones, serum enzymatic activities, and sperm quality parameters was determined using one-way analysis of variance (ANOVA) followed by the LSD test. Statistical significance was determined at $p < 0.05$. All analyses were performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

The body weight of voles did not differ significantly among the three groups ($F_{2, 32} = 0.574$, $p = 0.569$) (Fig. 1). Relative testis weight differed significantly among the three groups ($F_{2, 15} = 33.200$, $p < 0.001$). Relative testis weight was higher in the low dose TA group compared to the control and high dose TA groups ($p < 0.001$, for both comparisons; Fig. 2). Relative epididymis weight differed significantly among the three groups ($F_{2, 15} = 41.777$, $p < 0.001$). Relative epididymis weight was higher in the low dose TA group compared to the control and high dose TA groups ($p < 0.001$, for both comparisons; Fig. 3).

Figures 2–3. The effect of TA on the reproductive organ weight. Relative testis weight (2) and relative epididymis weight (3) of male *Lasiopodomys brandtii* provided low dose (3 mg/mL) and high dose (6 mg/mL) tannic acid (TA). Error bars indicate standard error. Same letters connect bars with no significant differences at $p < 0.05$ (n = 6). Note: Relative testis and epididymis weight were calculated as paired testes and epididymis weight (g) divided by body weight (g).
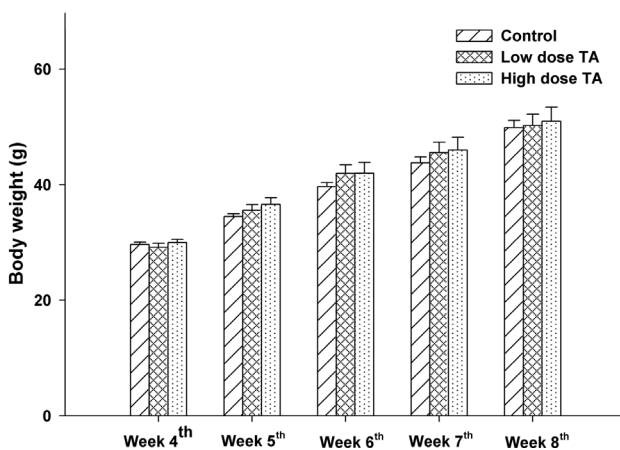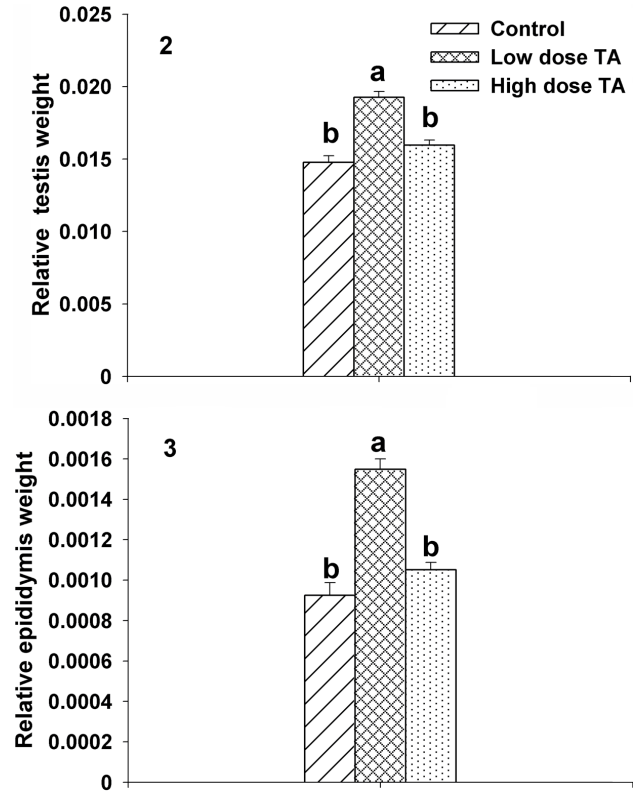

Figure 1. The effect of TA on body weight. The body weight of male *Lasiopodomys brandtii* provided low dose (3 mg/mL) and high dose (6 mg/mL) tannic acid (TA) from four weeks of age to eight weeks of age. Error bars indicate standard error (n = 6).

Sperm density differed significantly among the three groups ($F_{2, 15} = 5.080$, $p = 0.021$), with the low dose TA group having higher sperm density than the control group ($p = 0.006$) (Fig. 4). The sperm abnormality rate differed significantly among the three groups ($F_{2, 15} = 11.695$, $p = 0.001$), with the high dose TA group having higher sperm abnormality rates than the control and low dose TA groups ($p = 0.003$ and $p < 0.001$, respectively; Fig. 5). Sperm mortality rate followed the same trend, and also differed significantly among the three groups ($F_{2, 15} = 11.695$, $p = 0.001$), with the high dose TA group having higher sperm mortality rates than the control and low dose TA groups ($p < 0.001$, for both comparisons). Sperm mortality was also higher in the control group compared to the low dose group ($p = 0.034$) (Fig. 5).

The concentration of serum LH differed significantly among the three groups ($F_{2, 15} = 9.370$, $p = 0.002$), with the control group having lower serum LH than the low dose and high dose TA groups ($p = 0.001$ and $p = 0.015$, respectively; Fig. 6). The concentration of serum FSH also differed significantly among
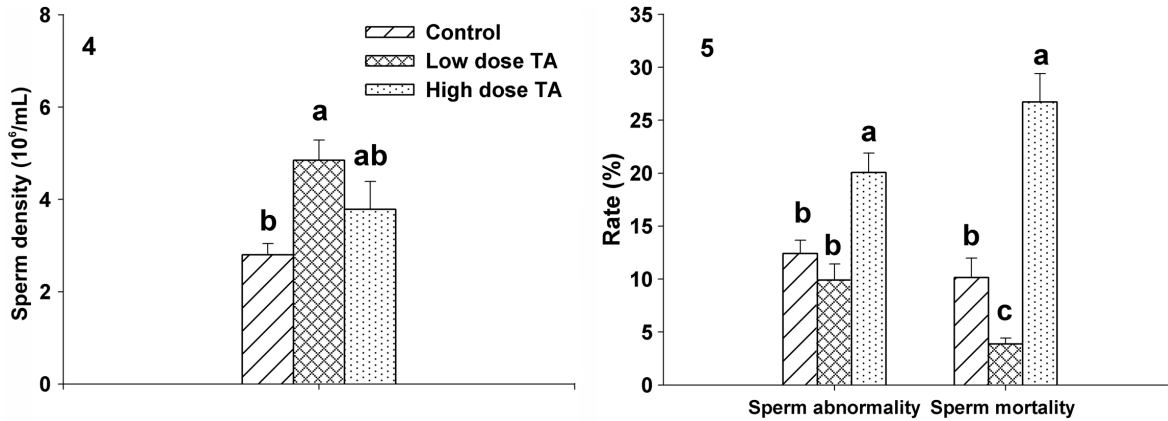
Figure 4–5. The effect of TA on the sperm quality. Sperm density (4), Sperm abnormality rate (5), and Sperm mortality rate (5) in the epididymis of male *Lasiopodomys brandtii* provided low dose (3 mg/mL) and high dose (6 mg/mL) tannic acid (TA). Error bars indicate standard error. Same letters connect bars with no significant differences at p < 0.05 (n = 6).
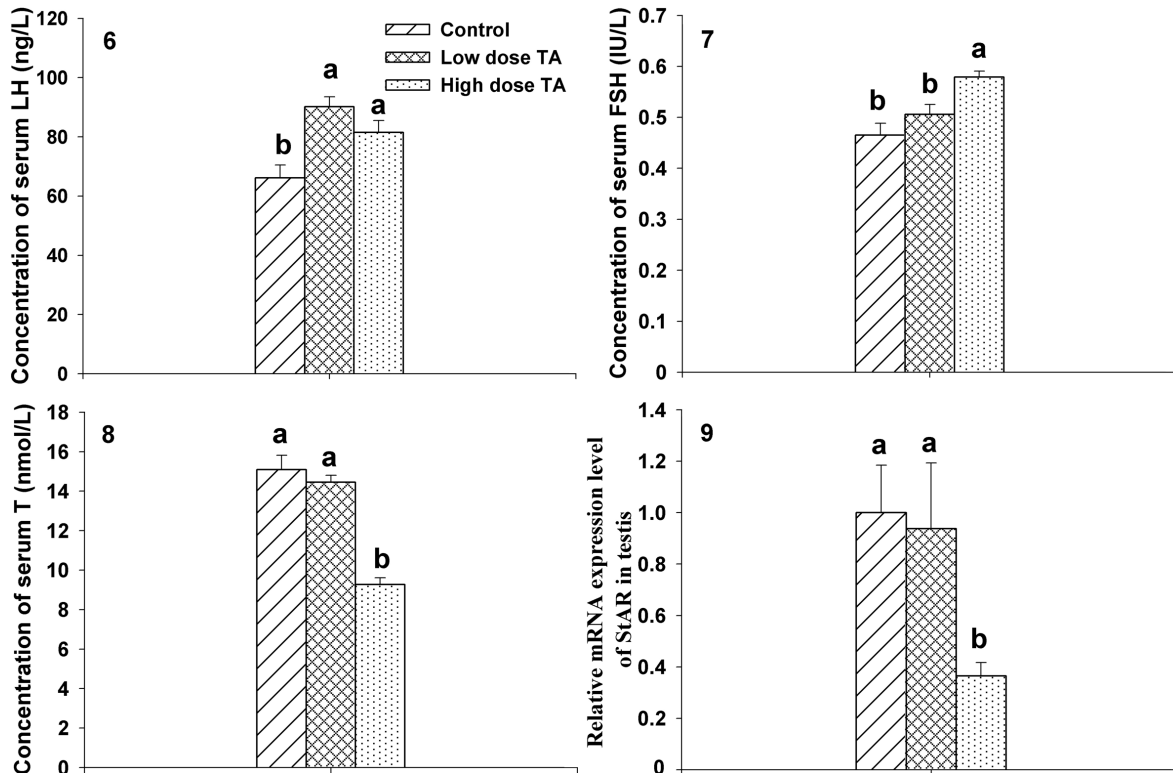

Figure 6–9. The effect of TA on the reproduction-related hormones concentration and StAR expression. Concentrations of luteinizing hormone (LH) (6), follicle-stimulating hormone (FSH) (7), and testosterone (T) (8) in the serum and relative mRNA expression levels of steroidogenic acute regulatory protein (StAR) (9) in the testes of male *Lasiopodomys brandtii* provided low dose (3 mg/mL) and high dose (6 mg/mL) tannic acid (TA). Error bars indicate standard error. Same letters connect bars with no significant differences at p < 0.05 (n = 6).

the three groups ($F_{2,15}$ = 9.511, p = 0.002), with the high dose TA group having higher serum FSH than the control and high dose TA groups (p = 0.001 and p = 0.014, respectively; Fig. 7). The

concentration of serum T differed significantly among the three groups ($F_{2,15}$ = 40.073, p < 0.001), with the high dose TA group having lower serum T than the control and low dose groups

(p < 0.001, for both comparisons; Fig. 8). The mRNA level of StAR also differed significantly among three treatment groups ($F_{2, 15}$ = 4.222, p = 0.035), with the high dose TA group having lower StAR mRNA levels than the control group (p = 0.015) and the low dose TA group (p = 0.044) (Fig. 9).
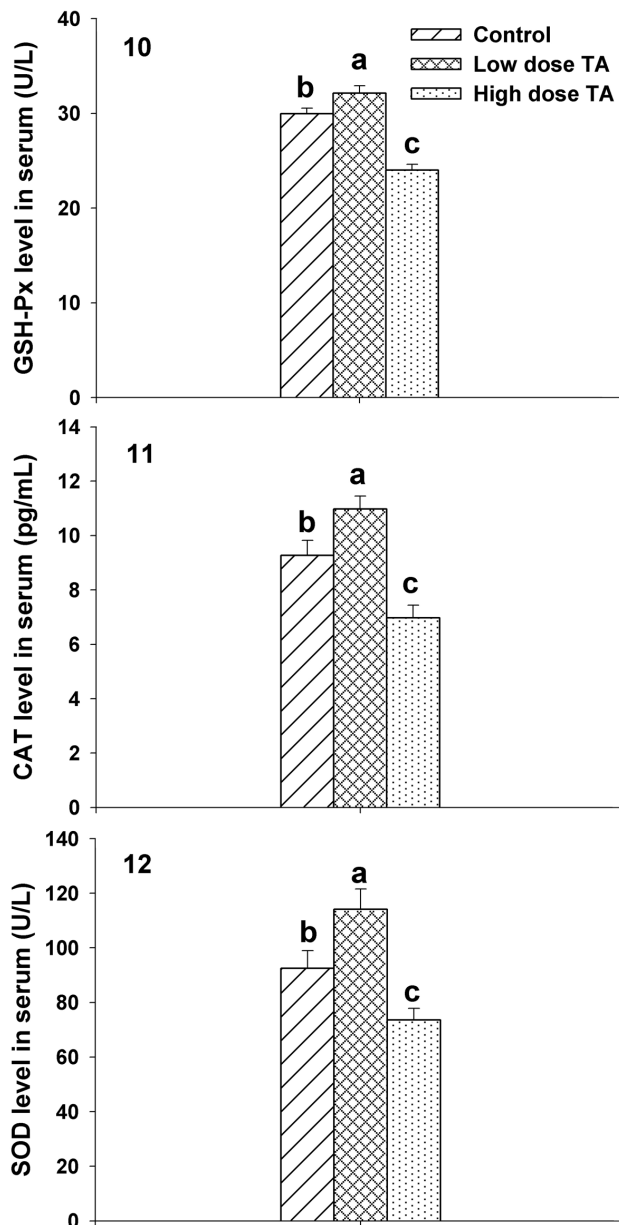
GSH-Px levels in serum differed significantly among the three groups ($F_{2, 15}$ = 40.822, p < 0.001), with the high dose TA group having lower GSH-Px activity than the control and low dose TA groups (p < 0.001, for both comparisons). Also, GSH-Px levels were lower in the control group compared to the low dose TA group (p = 0.034) (Fig. 10). Serum CAT levels followed the same trend, and also differed significantly among the three groups ($F_{2, 15}$ = 24.178, p < 0.001), with the high dose TA group having lower CAT levels than the control and low dose TA groups (p = 0.006 and p < 0.001, respectively). CAT levels were lower the control group compared to the low dose TA group (p = 0.028) (Fig. 11). Serum SOD levels differed significantly among the three groups ($F_{2, 15}$ = 10.755, p = 0.001), with the high dose TA group having lower SOD levels than the low dose TA and control groups (p < 0.001 and p = 0.047, respectively). Also, SOD levels were lower in the control group compared to the low dose group (p = 0.026) (Fig. 12).

The mRNA level of LC3A differed significantly among the three treatment groups ($F_{2, 15}$ = 12.718, p = 0.001), with the low dose TA group having lower LC3A mRNA levels than the control group (p = 0.010) and high dose TA group (p < 0.001; Fig. 13). The mRNA levels of Beclin1 also differed significantly among the three groups ($F_{2, 15}$ = 10.007, p = 0.002), with the low dose TA group having lower Beclin1 mRNA levels than the control group (p < 0.001) and high dose TA group (p = 0.015; Fig. 13).

Figure 10–12. The effect of TA on the content of anti-oxidative enzymes in serum. GSH-Px (10), CAT (11), and SOD (12) level in the serum of male *Lasiopodomys brandtii* provided low dose (3 mg/mL) and high dose (6 mg/mL) tannic acid (TA). Error bars indicate standard error. Same letters connect bars with no significant differences at p < 0.05 (n = 6).
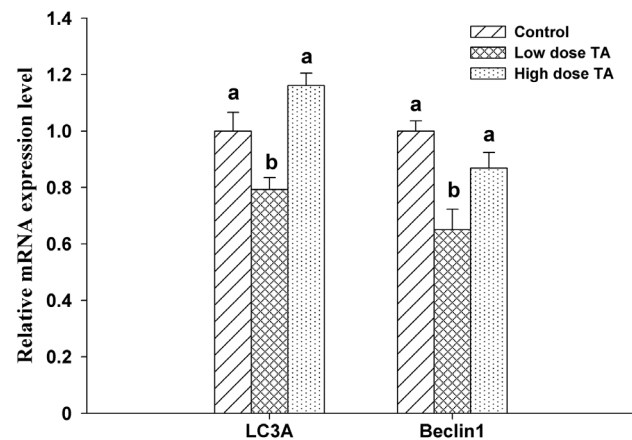

Figure 13. The effect of TA on the expression of autophagy-related genes. Relative mRNA expression levels of LC3A and Beclin1 in the testes of male *Lasiopodomys brandtii* provided low dose (3 mg/mL) and high dose (6 mg/mL) tannic acid (TA). Error bars indicate standard error. Same letters connect bars with no significant differences at p < 0.05 (n = 6).

DISCUSSION

Our study demonstrates that TA significantly affects the activity of antioxidative enzymes, sperm quality, reproductive organ weight, serum sex hormone, and autophagy in the testis

of male Brandt's voles. Drinking water containing TA for four weeks did not have any significant difference on body weight. Consistently, Brandt's voles supplemented with 3% TA in their diet for five weeks showed minimal variation in body weight to the control (Ye et al. 2016). Feeding 3.3% TA in the diet of Brandt's voles also did not significantly change their body mass (Chen et al. 2005b). Similarly, plateau zokors, *Eospalax baileyi* (Thomas, 1911) fed with tannic acid maintained their body weight (Lin et al. 2012). Thus, herbivores might have some physiological mechanism that allows them to adapt to certain doses of TA, allowing them to maintain body mass.

The relative testis and epididymis weight, serum luteinizing hormone, and the sperm concentration was highest in the low dose TA group in this study. Our previous study showed that giving tannic acid by intragastric administration increases serum T concentrations in plateau pikas, *Ochotona curzoniae* (Hodgson, 1858), and root voles, *Microtus oeconomus* (Pallas, 1776) (Dai et al. 2011). Compared to the control, tannins increase epididymal sperm concentrations and decrease abnormal sperm rates in rats (Türk et al. 2008). However, in the low dose TA group of our study, serum testosterone concentrations did not rise, which was not consistent with the relative increase in testis weight and sperm concentration, along with decrease in sperm abnormalities and sperm mortality. The reason for this phenomenon needs further research. In comparison, voles in the high-dose TA group had the lowest serum T and StAR mRNA levels, but the highest levels of both sperm abnormalities and sperm mortality, which tends to indicate that a sufficient amount of testosterone is vital for normal spermatogenesis (Sharpe 1994, Widyastuti et al. 2018). Thus, TA has a dose-dependent effect on the reproductive capability of male Brandt's voles. The lower dose of TA might enhance the development of reproductive organs of male Brandt's voles, whereas the higher dose damages reproductive function. Furthermore, both LH and FSH increased in the two TA-treated groups, possibly due to the negative feedback effect of testosterone on the HPG-axis pathway. Thus, the control group had the highest T concentrations.

SOD is mainly responsible for converting superoxide radicals to H_2O_2 and molecular oxygen. H_2O_2 is converted by GSH-Px and CAT into harmless water (Fridovich 1978, Imre et al. 1984, Hazelton and Lang 1985). Compared to the control, SOD, CAT, and GSH-Px activity in the low dose TA group of the current study concomitantly elevated. TA is considered to be a natural antioxidant capable of eliminating free radicals (Ye et al. 2016). Gallic acid (GA) is a natural hydrolyzed product of TA that rapidly and non-enzymatically carries out oxidization to generate large quantities of H_2O_2 in physiological solutions (Gil-Longo and González-Vázquez 2010). Concomitant induction of SOD, CAT, and GSH-Px by GA was also detected in rats (Hsu and Yen 2007). Therefore, certain doses of TA might enhance antioxidative defense and reduce oxidative stress in Brandt's voles. Similarly, CAT and GSH-Px activity increases in hepatic cell when adolescent Brandt's voles are treated with a

TA diet for five weeks (Ye et al. 2016). Furthermore, tannins in pomegranate juice were found to increase the plasma level of antioxidant enzymes (CAT and GSH-Px) in male rats (Türk et al. 2008). However, unexpectedly, in the high dose TA group of our study, CAT and GSH-Px activity significantly decreased compared to the control. Lower CAT and GSH-Px activity might cause the accumulation of excess H_2O_2, implying higher oxidative stress (Aitken and Roman 2008). Thus, the antioxidative effect of TA on male Brandt's voles might be dose-depended, with lower TA doses enhancing their ability to combat oxidative stress, while higher doses weaken defenses against oxidative stress.

Both spermatogenesis (Peltola et al. 1994) and Leydig cell steroidogenesis (Quinn and Payne 1984, Chen et al. 2005a) in the testis are vulnerable to oxidative stress. An array of antioxidant enzymes ensures that the spermatogenic and steroidogenic functions of testis are not impacted by oxidative stress (Aitken and Roman 2008). For example, factors that induce oxidative stress, such as the environmental toxicant fluoride (Barbier et al. 2010), cause spermatogenic dysfunction in human beings and experimental animals (Long et al. 2009). The present study showed that the high dose TA group, which had lower SOD, CAT, and GSH-Px activity, also had the lowest concentration of serum T and StAR mRNA level, as well as the highest rate of sperm abnormality and mortality. In comparison, the low dose TA group had higher SOD, CAT, and GSH-Px activity, and the higher relative testis weight and sperm density, along with lower sperm mortality. While the actual levels of SOD, CAT, and GSH-Px in the testis were not known in the current study, the levels of these anti-oxidative enzymes were detected in serum and approximately reflect the anti-oxidative levels of the testis. Thus, we concluded that TA influences the spermatogenic and steroidogenic functions of the testis of Brandt's voles by regulating the level of oxidative stress. On the basis of the findings of previous studies, in which the authors quantified tannin content in the preferred food plants of Brandt's voles (Yin et al. 2017) and investigated the amounts of plant food that voles consumed each day (Wan et al. 2001), we assumed that the daily tannin intake of pubertal and adult voles in the grasslands of Inner Mongolia from May to August probably exceeds that administered to voles in the high-dose TA group of the present study. In the current study, TA was administered to voles for four weeks, from four weeks in age (pubertal stage) to eight weeks in age (adult stage). Thus, our results might reflect the response of wild male voles to TA that were born in the proximate breeding season. Therefore, reproduction by male voles born in the proximate year might be suppressed by secondary plant metabolites, which would constrain the size of the vole population. Reproduction by male Brandt's voles born in the proximate year is consistently suppressed, whereas male Brandt's voles born in previous years participate in reproduction (Ren et al. 2016). Fierce competition among male voles was considered to be the reason for this phenomenon (Ren et al. 2016). Our study proposed that plant secondary metabolites might also

contribute to the reproductive suppression of male Brandt's vole born in the proximate breeding season, besides social factors in the vole community.

Compared to the control, the mRNA levels of LC3A and Beclin1 significantly decreased in the testis of low dose TA group voles in this study. Thus, low dose TA might reduce autophagy activity in the testis of adolescent Brandt's voles, supporting our hypothesis that tannins mediate autophagy. The autophagy pathway represents an alternative approach to achieving more desirable antioxidative effects, without perturbing the redox signaling pathway, through clearing damaged proteins and organelles generated by diverse oxidants (Giordano et al. 2014). Compared to the control, anti-oxidative defense levels (level of SOD, CAT, and GSH-Px) of the redox signaling pathway were higher in the serum of voles in the low dose TA group, which contrasted with the level of autophagic activity in these two groups. Thus, the redox signaling pathway and autophagy activity complement each other towards maintaining defense levels against oxidative stress. Therefore, in the low dose group, TA enhanced the activity of components in the redox signaling pathway, indirectly reducing autophagy. However, compared to the control, the lower antioxidative enzyme activity of the high dose TA group did cause autophagy in the testis to increase significantly. Oxidative stress mediates autophagy, with high oxidative stress promoting autophagy (Slater et al. 1995, Lee et al. 2012). Therefore, we speculated that TA also has a direct inhibitory effect on autophagy in the testis. Deficient autophagy could impair spermatogenesis (Wang et al. 2014). Overall, the expected stronger autophagy activity corresponding to lower anti-oxidative levels in the high TA group was inhibited by TA. This phenomenon resulted in relatively deficient autophagy, and contributed to lower spermatogenic and steroidogenic functions in the high dose group.

In summary, our study demonstrated that lower doses of TA enhance anti-oxidative levels and reduce autophagy in the testis, in turn, enhancing the reproductive capability of male Brandt's voles treated with TA from the pubertal stage. In comparison, higher dose of TA cause anti-oxidative levels to decrease, thus impairing spermatogenic and steroidogenic functions. TA might mediate autophagy in the testis through indirectly regulating anti-oxidative levels and directly inhibiting autophagy. Our study showed that TA mainly affects the reproductive function of male Brandt's voles by regulating anti-oxidative levels, with the direct effect of TA on autophagy impacting the reproductive capacity of Brandt's voles. Our study provides new insights on the mechanism by which plant secondary metabolites affect the reproduction of herbivores and how plant secondary metabolites regulate herbivore populations.

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