1 **Effects of dietary tannic acid on the growth, hepatic gene expression, and**
2 **antioxidant enzyme activity in Brandt's voles (*Microtus brandti*)**

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17 Abbreviations: TA, tannic acid; HMGCR, 3-hydroxy-3-methylglutaryl coenzyme A
18 reductase; Trf, transferrin; SOD, superoxide dismutase; CAT, catalase; GPx,
19 glutathione peroxidase.

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Abstracts

This study was designed to investigate the physiological and biochemical responses of Brandt's voles to the persistent presence of dietary tannic acid. The diet for animals in the experimental group was supplemented with 3% dietary tannic acid for five weeks. The control group received a commercial lab chow. No significant differences were detected in body weight, organ (heart, kidney, and liver) weights, and organ parameters between animals from two groups. However, voles in the experimental group had significantly higher daily food intake, increased contents of proline and histidine in saliva and feces after protein hydrolysis, and elevated hepatic expression of transferrin than the control. Our results suggested the existence of adaptive strategies developed in Brandt's voles to overcome the adverse effects of dietary tannic acid. (1) Food consumption was increased to satisfy their nutritional demands. (2) The secretion of tannic-acid-binding salivary proteins was promoted. (3) The absorption of iron was enhanced. These alterations contributed to neutralize the negative effects of tannic acid and maintain body mass in animals supplemented with tannic acid. As the result of the consumption of tannic acid, hepatic expression of 3-hydroxy-3-methylglutaryl coenzyme A reductase was significantly decreased, while the overall potential of the antioxidant system, characterized by increased hepatic enzymatic activities of catalase and glutathione peroxidase, was enhanced. Our results also implied the involvement of tannic acid in the regulation of lipid metabolism and oxidative stress in voles.

Keywords: tannic acid, food intake, gene expression, antioxidant enzymes

1. Introduction

Tannic acid (TA), one member of hydrolyzed tannins abundant in a wide variety of plants, is a model compound representative of polyphenols with high molecular weight that occur mainly in plant-derived feeds, food grains, and fruits. Ingestion of TA is known to cause various types of effects in animals. Having the ability to form complexes with proteins, starch, and digestive enzymes, TA is often considered to be an anti-nutritional factor for many animals (Chung et al., 1998) and has adverse effects of reducing food efficiency and growth rate (Mansoori and Acamovic, 2007; Lee et al., 2010). On the other hand, its beneficial biological effects were also observed. It has been reported to possess antioxidant (Wu et al., 2004; Andrade et al., 2005; Gülçin et al., 2010; Bouki et al., 2013), anti-mutagenic (Ferguson, 2001; Chen and Chung, 2000) and anti-carcinogenic (Nepka et al., 1999; Tikoo et al., 2011) properties. It is also used as an effective antioxidant in food industry (Maqsood et al., 2012).

Brandt's voles (*Microtus brandti*) are non-hibernating herbivores that mainly inhabit the grasslands of Inner Mongolia of China, the Republic of Mongolia, and the region of Beigaer Lake in Russia, where winter lasts for more than 5 months (Li and Wang, 2005). The Brandt's vole is one of the smallest (40-50 g) strictly herbivorous mammals and feeds on both monocotyledons and dicotyledons. Despite its small size, the Brandt's vole consumes about 40 g fresh grass per day. Under cold conditions, the amount of food it consumes daily can even exceed its body mass (Zhang and Wang, 1998). It is a dominant pest rodent in North China. During the past decade, much

work has been done to investigate the role of cold acclimation on energy metabolism and the regulation of body mass in Brandt's voles (Li et al., 2001; Li and Wang 2005; Zhang and Wang, 2006 and 2007; Zhang et al., 2009; Li et al., 2010; Zhang et al., 2015). Whereas available information about the adaptive changes in morphology and physiology in response to nutritional factors, which are critical for their survival, are limited in Brandt's voles. Several researchers studied the influence of dietary fibre on the food intake and digestibility in Brandt's voles. Pei et al. (2001) reported the digestive responses of Brandt's voles to an increased content of dietary fibre in terms of the digestive performance, gut morphology, and rate of passage of digesta. Their results indicated that Brandt's voles could compensate the poor-quality diet physiologically by the means of increasing food intake. Similarly, increased food intake and decreased digestibility were observed in Brandt's voles fed with high-fibre diet (Song and Wang, 2006; Zhao and Wang, 2007). It has been reported that TA constitutes the main phenolics in the Brandt's vole's daily food, accounting for 5% of the dry food (Li et al., 2007). However, little is documented about the physiological modifications that occur as a consequence of TA consumption in Brandt's voles.

As is known, TA is one of the most common anti-nutritional factors in food associated with poor utilization of protein. It has also been recognized as the inhibitor of non-heme-iron absorption (Kalgaonkar and Lönnnerdal, 2008; Lee et al., 2010; Jin et al., 2009; Lavin et al., 2010; Andrews et al., 2014; Hurrell and Egli, 2010; Jaramillo et al., 2015). We assumed that in order to avoid the possible deficiency of nutrients (protein and iron, especially) the Brandt's voles must develop corresponding

mechanisms in their daily interactions with dietary TA to counteract its adverse effects. In this study, we investigated the influences that 3% dietary TA (which is within the average content of TA in Brandt's voles' natural foods) might have on voles in terms of: (1) average daily food intake, (2) the profile of amino acids in saliva and feces after protein hydrolysis, (3) hepatic expression of HMGCR (3-hydroxy-3-methylglutaryl coenzyme A reductase, the major rate-limiting enzyme for *de novo* cholesterol synthesis in the liver) and Trf (transferrin, produced especially by hepatocytes and critical for iron uptake), (4) activities of antioxidant enzymes represented by SOD (superoxide dismutase), CAT (catalase) and GPx (glutathione peroxidase). In this way, we try to explain how the Brandt's voles manage to maintain their normal growth status in the continuous presence of dietary TA.

2. Material and Methods

2.1 Animals

Thirty two four-week-old Brandt's voles, weighing 23.6 ± 1.39 g, were used in the present study. They came from a breeding colony of Brandt's voles maintained at the College of Bioscience and Biotechnology, Yangzhou University, Yangzhou, Jiangsu Province, China. Founder animals in the colony were originally captured in the wild in Xilinhaote League, Inner Mongolia Autonomous Region of China. Animals were kept individually in wire-bottomed cages under a 12-hour light/dark cycle and controlled temperature (22-25°C) with a relative humidity of 40-60%. Drinking water and commercial chow pellets (containing no tannic acid) for growing and breeding rabbits were provided *ad libitum*. The nutrient contents of the food were as follows:

crude protein $\geq 15\%$, crude fat $\geq 4\%$, crude fiber: 10%-15%, ash: $\leq 8\%$, calcium: 1.0-1.8%, and phosphorus: 0.6-1.2%. All experimental procedures were performed following the “Guide for Care and Use of Laboratory Animal” of the Comparative Medical Centre of Yangzhou University (a registered facility for supervising experiments on laboratory animals), and in accordance with the local Animal Care and Use Committee.

2.2 Animal grouping and sample collection

Brandt's voles were divided randomly into two groups (control group and TA-treated group, respectively) with sixteen animals in each group, half male and half female. Voles in the control group were fed with commercial lab chow, obtained from the Comparative Medical Centre of Yangzhou University. Animals in the TA-treated group were fed with a diet containing 3% TA (molecular weight 1701.2), which was purchased as dry power from Sinopharm Chemical Reagent Co., Ltd., Shanghai, China. TA was added to the standard diet at the expense of the whole diet. All the voles were monitored for general health and weighed individually each day. Food intake was recorded daily for calculation of average food intake by each vole. The treatments lasted for continuous five weeks. On the 36th experimental day, feces uncontaminated by urine were collected and whole saliva of individual vole was obtained. Saliva production was induced with an intraperitoneal injection of pilocarpine (Sigma, 5 mg per kg body weight), dissolved in 1 mL 9% (m/v) sodium chloride, as described by Muenzer et al. (1979). Saliva was collected directly from the mouth with a micropipette. Both the feces and saliva samples were frozen

immediately at -80°C and used for amino acid analysis. Saliva samples were also separated on 10% SDS-Tris-glycine polyacrylamide gels to get a general profile of saliva proteins. Animals were sacrificed after being anesthetized with 50 mg sodium pentobarbital per kg body weight. Body weight, heart, kidney (both sides) and liver weight were recorded. Liver tissue was cut into pieces, snap-frozen in liquid nitrogen for subsequent analyses.

2.3 Determination of amino acid

Tannin-binding salivary proteins, mainly proline-rich proteins and histatins (rich in histidine), have been reported for many species of rodents and lagomorphs, such as mouse, root vole, rabbit, mountain hare, and North American pika (Shimada 2006). These salivary proteins were reported to be one of the important means by which herbivores minimize the effects of dietary tannins. The presence or absence of these proline- and histidine-rich salivary proteins in the Brandt's vole has not been documented. In this study, the composition of total amino acids in feces and saliva samples was analyzed using an amino acid analyzer (L-8900, Hitachi, Japan) according to the Chinese Standard GB/T 5009.124-2003 (2004). Briefly, samples were hydrolyzed in 6 N HCl solution at 110°C for 22 hours after 10 min of nitrogen blowing in vacuum-sealed glass tubes. For each tube, 1 mL hydrolysate was passed through a 0.22 µm membrane filter and aliquoted into two vials. From each vial, 20 µL of the hydrolysates were injected using an auto-sampler. Mixed standard amino acids were analyzed before sampling. The amino acids were identified and quantified by comparing peak profiles of the tested samples with profiles of standard amino

acids.

2.4 PCR amplification for *HMGCR* and *Trf* gene in the liver tissue

Protein sequences for *HMGCR* and *Trf* from various species were obtained from the EMBL database at <http://srs.ebi.ac.uk/>. After alignment of the protein sequences with the ClustalW 2 program (<http://www.clustal.org/>), consensus-degenerate PCR primers were designed according to the CODEHOP strategy (<http://blocks.fhcrc.org/codehop.html>). All the PCR-related reagents and commercial kits used in this assay were obtained from Takara Biotech Co., Ltd., Dalian, China.

In brief, total RNA was isolated from the liver tissue with Trizol reagent and complementary DNA (cDNA) was synthesized from 2 µg of total RNA from each sample using the cDNA synthesis Kit according to the manufacturer's instructions. A touchdown PCR was then executed under the following conditions: a pre-denaturation step of 5 min at 95°C, followed by 15 cycles of touch down PCR (30 s at 95°C, then an initial annealing temperature of 61°C with a temperature decrement of 1°C following each cycle and treated for 2 min at 72°C thereafter). From the 16th cycle the annealing temperature of 47°C was kept fixed for the next 15 cycles, ending with an extension period of 10 min at 72°C. The PCR products with expected fragment length were ligated into pGEM-T vector (Promega, Madison, USA) and transformed to *E. coli* cells (TOP10 strains). Sequencing of the positive clones was performed on an ABI 3130 DNA Sequencer (Applied Biosystem). 3' RACE (rapid amplification of cDNA ends) was also performed for *HMGCR* gene by using a commercial 3'-Full RACE Core Set Ver.2.0 kit. These derived sequences for *HMGCR* gene and *Trf*

gene were used to design the real time primers using Primer 3.0 software. All primers were synthesized by Sangon Biotech Co., Ltd., Shanghai, China. The complete sequences of primers used in this study were listed in Table 1.

2.5 Quantitative Real-time PCR (qRT-PCR)

The relative mRNA expression levels of *HMGCR* gene and *Trf* gene in the liver tissue were determined by qRT-PCR method. β -actin was used as internal reference gene. qRT-PCR, in a 10 μ L reaction volume with 0.1 μ M of primers, was performed in triplicate for each reaction using 2 $\times$ SYBER Premix Ex Taq II (Takara, Biotech Co., Ltd., Dalian, China). The PCR conditions were as follows: 95°C 5 min for initial denaturation, 40 cycles of denaturation at 95°C for 30 sec and 60°C for 30 sec. qRT-PCR was performed in an ABI PRISM 7700 sequence detection system (Applied Biosystems). The specificity of PCR amplification was demonstrated by melting curve analysis post PCR reactions as well as sequencing. Data were processed by the $2^{-\Delta\Delta CT}$ method as described previously (Livak and Schmittgen, 2001), presented in gene expression normalized to the β -actin gene, and relative to the samples from the control group. Mock reverse transcription and no template controls were set to monitor the possible contamination of genomic and exogenous DNA at both RT and PCR levels.

2.6 Protein extraction and Western blot analysis

Western blot analysis was conducted to analyze the expression of HMGR, Trf, SOD-1 (also known as Cu/Zn SOD, which represents the majority of total SOD activity in a eukaryotic cell) and GPx-1(cytosolic glutathione peroxidase, which is the

major enzyme responsible for removing H₂O₂ in the family of selenium-dependent peroxidases) at the translational level. Total cellular and nuclear proteins were extracted from 100 mg frozen liver tissue using Radio Immunoprecipitation Assay Lysis Buffer containing 1 mM PMSF (phenylmethanesulfonyl fluoride) as protease inhibitors (Beyotime Institute of Biotechnology, Shanghai, China). Lysates were cleared by centrifugation (14,000×g, 10 min) at 4°C and the supernatant was stored as aliquots at -80°C. Protein concentration in the samples was determined using an Enhanced BCA Protein Assay Kit (Beyotime Institute of Biotechnology, Shanghai, China). Western blot analysis for HMGCR (ab180615, Abcam, UK, diluted 1:500), Trf (ab1223, Abcam, UK, diluted 1:1000), SOD-1 (BS6057, Bioworld, USA, diluted 1:500), and GPx-1 (BS6090, Bioworld, USA, diluted 1:500) was carried out according to the recommended protocols provided by the manufacturers. The protein content in total cellular lysates was normalized with β-actin (BSAP0060, Bioworld, USA, diluted 1:10,000). These antibodies were all developed from rabbit.

Briefly, equal amounts (30 μg) of boiled protein (3 min at 100°C) were loaded onto reducing 10% SDS-Tris-glycine polyacrylamide gels and then electroblotted to nitrocellulose membrane (ImmobilonTM-P, Millipore, Watford, UK) using a Bio-Rad CriterionTM blotter. The molecular weights of the bands were evaluated by comparison with a molecular weight marker (Takara Biotech Co., Ltd., Dalian, China) that was processed simultaneously. In immunodetection, the membranes were blocked with 5% nonfat milk in TTBS (20 mM Tris, pH 6.8, 150 mM NaCl, 0.05% Tween 20) overnight at 4°C. Following incubation with primary antibody in TTBS at room

temperature for 1 h, the immunoblot was washed with TTBS (4×15 min) and then incubated with ECLTM-horseradish peroxidase-conjugated sheep-anti-rabbit IgG (Amersham Biosciences, 1:10,000 dilution) for 1 h. Finally, all the blots were washed three times with TTBS and chemiluminescent substrate (SuperSignal, Pierce, Rockford, USA) was applied for visualization of the corresponding bands. The chemiluminescent image was captured by autoradiography. Optical densities of individual bands were measured with Image-Pro Plus 6.0 software on the scanned image with background subtraction and the expression of protein was normalized to β -actin.

2.7 Assays of enzymatic activities

In vitro enzymatic assays for SOD, GPx and CAT were performed using commercial enzyme assay kits (A001-1, A005, and A007-2 from Nanjing Jiancheng Bioengineering Institute, Nanjing, China) in accordance with the manufacturer's instructions. Briefly, liver tissues were weighed and homogenized in nine volumes of ice-cold 0.86% physiological saline using a homogenizer. The lysate was centrifuged (10,000×g, 10 min, at 4°C) and the supernatant was recovered, aliquoted, and kept on ice until analysis. Concentration of the soluble protein present in supernatant samples was determined using the method of Bradford with bovine serum albumin (BSA) as a standard. Total SOD activity was measured by inhibition of the oxidation of hydroxylamine. The method is based on the xanthine-xanthine oxidase system, which generates superoxide anions (O_2^-), these in turn, lead to the oxidation of hydroxylamine. The oxidation can be inhibited by the SOD present in the supernatant

and the changes in absorbance can be monitored at 550 nm. The amount of SOD in the supernatant sample sufficient to inhibit the rate of oxidation of hydroxylamine by 50% was defined as one unit (U) of activity, and expressed as units per mg protein. The GPx activity was determined by the method of Hafeman et al. (1974). Using hydrogen peroxide (H_2O_2) as substrate, this indirect method is based on the conversion of glutathione (GSH) to oxidized glutathione (GSSG) by GPx. The GSH level is measured by using 5,5'-dithiobis (2-nitrobenzoate), leading to the production of 5-thio-(2-nitrobenzoic acid) which shows absorption at 412 nm. An enzyme unit was defined as a decrease of one μM in the concentration of GSH per minute per mg protein after the non-enzymatic reaction was subtracted. In both assays, the volume of supernatant added to the assay mixture was adjusted to make sure that the inhibiting rate was between 15% and 55%. Thus, the rate of oxidation of hydroxylamine and GSH was proportional to enzymatic activities in the samples. The activity of CAT was determined following the breakdown of H_2O_2 using the spectrophotometric method described by Aebi (1984). The decomposition of H_2O_2 was measured by the decline in absorbance at 240 nm. The specific activity was expressed as unit per mg protein, where one unit of catalase converted one mol of H_2O_2 per min. Assays were done in triplicate. For each homogenate sample, two different aliquots were used.

2.8 *Statistic analysis*

Statistic analysis was performed with the SPSS 13.0 software package. All data were presented as mean $\pm$ SD. Student's t-test was employed for statistical analysis and statistical significance was defined as a *P* level below 0.05.

3. Results

3.1 Effects of TA on body weight, organ weight, and food intake

No significant differences were detected in body weight, organ (liver, heart and kidney) weights and organ parameters (organ weight/body weight) between two groups. However, TA did have a significant effect on the average daily food intake (Table 2). Voles in the TA-treated group had significantly higher daily food intake than control animals ($P < 0.0001$) during the whole experiment except the four days since the beginning of the experiment.

3.2 Analysis of amino acids

Compared to the control animals, Brandt's voles from the TA-treated group had significantly higher contents of proline (Pro) and histidine (His) in feces and saliva samples after protein hydrolysis ($P < 0.01$) (Table 2).

Results from the SDS polyacrylamide gel electrophoresis (SDS-PAGE) revealed that saliva samples from two groups had similar protein compositions. An extra band near 50 KDa was detected in saliva samples from TA-treated animals (Fig.1).

3.3 Amplification of *HMGCR* gene and *Trf* gene in the liver tissue

Using a pair of consensus-degenerate hybrid primers, HMGCR-up and HMGCR-down, a 2282-bp fragment was amplified. By using HMGCR-qPCRup as the specific upstream primer in 3'RACE, we identified a 2631-bp *HMGCR* mRNA sequence (GenBank KU522123), which contained an open reading frame of 876 amino acids. The coding region of *HMGCR* gene in the Brandt's vole shared a high degree of sequence similarity with known HMGCR proteins in rodents: 95% identity

to Chinese hamster (*Cricetulus griseus*) and golden hamster (*Mesocricetus auratus*), 94% identity to house mouse (*Mus musculus*) and Norway rat (*Rattus norvegicus*), 89% identity to domestic guinea pig (*Cavia porcellus*), naked mole-rat (*Heterocephalus glaber*), and thirteen-lined ground squirrel (*Spermophilus tridecemlineatus*).

A single strong band was observed after amplification with degenerate primer sets of Trf-up and Trf-down. Sequencing results revealed that the corresponding nucleotide sequence was of 427 bp encoding 141 AA residues (GenBank KU522124), which showed high degree of similarity with known Trf proteins from the rodents: 86% identity to Chinese hamster (*Cricetulus griseus*), 79% identity to house mouse (*Mus musculus*), 78% identity to Norway rat (*Rattus norvegicus*) and woodchuck (*Marmota monax*), 76% identity to naked mole-rat (*Heterocephalus glaber*), 73% identity to thirteen-lined ground squirrel (*Spermophilus tridecemlineatus*) and rabbit (*Oryctolagus cuniculus*), and 70% identity to guinea pig (*Cavia porcellus*).

3.4 Hepatic expression of *HMGCR* gene and *Trf* gene

The mRNA abundance of target genes was quantitated with qRT-PCR. The two specific primer sets, Trf-qPCRUup and Trf-qPCRdown, HMGCR-qPCRUup and HMGCR-qPCRdown, led to the amplification of PCR products (182bp and 238bp for *HMGCR* and *Trf*, respectively). In the liver of Brandt's voles treated with 3% dietary TA, *HMGCR* gene, which encodes HMGCR protein responsible for cholesterol biosynthesis, was found to be significantly down-regulated, while *Trf* gene, which

encodes protein involved in the transportation of iron, was significantly up-regulated (Fig. 2).

3.5 Hepatic level of HMGCR, Trf, SOD-1, and GPx-1

In line with hepatic mRNA abundance, results from western blot analysis (Fig. 3) confirmed that the HMGCR content in the whole lysates was significantly lower in the liver of animals from the TA-treated group, while the Trf content was significantly higher. Dietary TA also increased the contents of two antioxidant enzymes. In TA-treated voles, the hepatic GPx-1 content was significantly higher than control ($P < 0.01$), while the hepatic SOD-1 level was elevated without reaching the statistical difference ($P = 0.068$).

3.6 Responses of enzymatic activities to dietary TA

The activities of antioxidant enzymes in the homogenates of liver tissue were compared between animals from two groups (Fig. 4). TA-treated voles had significantly higher level of total GPx activity than the control. Though not significantly, values of the CAT activity were also higher in TA-treated voles. However, no statistical differences in the level of total SOD activity were detected between different groups.

4. Discussion

In the present study, Brandt's voles in the TA-treated group were supplemented with 3% TA in their diet for five weeks, which aimed to simulate the physiological conditions involved in long-term interactions with dietary TA. No significant differences were detected in body weight, organ (liver, kidney, and heart) weights,

331 and organ parameters between TA-treated voles and the control. However, an
332 enhancing effect of dietary TA on food intake was observed. In view of the published
333 reports, the effects dietary TA exerted on food intake in rodents were various. Many
334 researchers reported a reduction in food intake as a result of the astringent flavor TA
335 possesses (prairie vole, Meyer and Richardson, 1993; Japanese wood mouse, Shimada
336 and Saitoh 2003; reed vole, Li et al., 2007). Quite the contrary, earlier studies
337 observed two- to three-fold increased food intake (Innes and Millar, 1981; Lindroth
338 and Batzli, 1984). At the same time, evidence has also been presented that dietary TA
339 has no influence on food intake (Yugarani et al., 1992; Park et al., 2002; Afsana et al.,
340 2004). The influences dietary TA exerted on animals may vary depending on the
341 characteristics of the ingesting animal, the concentration of dietary TA, the protein
342 level of the diet, and possible interactions between these factors. It has been revealed
343 that the anti-nutritional effects of tannins are partly due to their complexation with
344 dietary proteins, digestive enzymes, and endogenous proteins, which reduces the
345 quality of available forage and results in a drain on the supply of nitrogen in animals
346 (Bhat et al. 2013). Results from previous studies also showed that ingestion of
347 tannin-rich diets reduced protein digestibility in prairie vole (Meyer and Richardson
348 1993), mule deer, domestic sheep, and black bears (Robbins et al. 1991). In this study,
349 the growth rate of Brandt's voles in TA-treated group was not suppressed. The
350 absence of depressed growth parameters indicated that dietary TA did not markedly
351 reduce the absorption of nutrients essential to growth. We suggested that TA-treated
352 voles might overcome the reduced efficiency of protein utilization by increasing food

consumption to maintain their body mass.

Saliva is the first line of defense against TA. Previous studies have revealed that consumption of tannins could induce the secretion of salivary proline-rich proteins and histatins in rat, mouse and root vole (Shimada 2006), which bind to tannins, form indigestible complexes to escape both enzymatic and microbial degradation, and are excreted in the feces (Skopec et al, 2004). In this study, significant increases of total Pro and His contents in saliva and feces samples after protein hydrolysis were observed in TA-treated voles. Our results provided indirect evidences for the presence of these effective precipitators in the Brandt's voles acting as a post-ingestive countermeasure against dietary TA. The extra protein band detected in saliva samples from TA-treated voles also suggested that the inducible secretion of salivary proteins was one of the key elements in mitigating the negative effects caused by TA. The mechanism to save dietary essential amino acids at the cost of the improved excretion of nonessential ones, such as Pro and His, is effective to protect animals from reduced assimilation of nutrients and practically useful for Brandt's voles that ingest TA as a persistent ingredient of their natural diets.

While the chelating property of TA due to the presence of galloyl residues enabled it to bind iron with high affinity, we speculated that a compensatory mechanism might be activated to ensure the enhanced transportation and/or absorption of iron. To further understand the interactions between dietary TA and the transportation of iron, expression of *Trf* mRNA as well as hepatic level of the Trf protein were evaluated. Our results demonstrated that hepatic Trf was significantly up-regulated at both the

transcriptional and translational levels in the presence of dietary TA vs in its absence, which were consistent with previous studies that documented significantly enhanced Trf synthesis/expression in cases when iron depletion might be caused (Kralova et al., 2008; Gkouvatsos et al., 2012). As is known that Trf-mediated uptake of iron represents the primary means for the uptake of non-heme iron by almost all cell types (Wallander et al., 2006). Most likely, dietary TA that reached in the stomach and intestines promoted the expression of Trf to enhance the absorption of iron. In this regard, we speculated that more iron ions could be bound by Trf rather than by the TA molecules, which allowed for greater amounts of iron available for absorption. It is worthy to note that Trf has also been revealed to be a multi-tasking protein. Besides being an iron-carrier, it was also considered to act as a growth factor or cell growth inducer (Gomme et al., 2005). In human, low levels of Trf was found to be associated with an increased incidence of infection and growth retardation (Hayashi et al., 1993). The significantly up-regulated expression of Trf, along with significantly increased food intake could be responsible for the lack of any retardation of growth in TA-treated voles in the present study.

An inhibitory effect of dietary TA was observed in hepatic *HMGCR*. The down-regulated expression of *HMGCR* gene was associated with cholesterol-lowering effect (Zhang et al., 2013) and hypolipidemic activities (Fan et al., 2013). The significantly reduced hepatic level of *HMGCR* transcript and HMGCR protein proved direct evidence in the Brandt's vole that dietary TA could regulate the expression of this key gene involved in cholesterol metabolism (Park et al., 2002), which might be

crucial for the dynamic balance of lipid metabolism. Further studies involving the high-fat diets were needed to clarify the beneficial effects TA might have on the lipid profile of Brandt's voles.

Cellular antioxidant defenses against oxidative stress involve antioxidant enzymes such as SOD, CAT and GPx. These enzymes belong to the primary antioxidant defense mechanisms which protect cells from the damages caused by the overproduction of reactive oxygen species (ROS) and reduce oxidative stress (Weydert and Cullen, 2010). In this study, no marked changes in total SOD activity were detected between voles from two groups. However, a significant increase in total GPx activity was observed in TA-treated voles. Though not significantly, values of the CAT activity were also elevated in TA-treated animals. It is well known that SOD is mainly responsible for the conversion of superoxide radical into H_2O_2 and molecular oxygen. No diet-related changes detected in the SOD activity might result from the unchanged production of superoxide. TA is commonly considered to be a natural antioxidant capable of scavenging the free radicals. It was reasonable that endogenous antioxidant defenses were not elevated in the presence of generous amounts of exogenous antioxidants. The role of GPx and CAT is to convert H_2O_2 to the harmless water. As they degrade the same kind of substrates, the activities of GPx and CAT are often related (Srikumar et al., 2006; Faria et al., 2007; Pattanayak and Mazumder, 2011). The elevation of their activities suggested that larger amounts of H_2O_2 were being generated. Previous reports documented that gallic acid (GA), one natural product after the hydrolysis of TA, was rapidly and non-enzymatically

oxidized generating large quantities of H₂O₂ in physiological solutions (Gil-Longo and González-Vázquez, 2010). Concomitant inductions of SOD, CAT and GPx were also reported in studies investigating health protective effects of GA (Hsu and Yen, 2007). To our best knowledge, there are no reports yet available about TA autoxidation. Our present results suggested that the increase in the activities of GPx and CAT was most likely induced to remove H₂O₂ produced by TA autoxidation rather than by SOD. To testify the hypothesis that H₂O₂ formation was enhanced at the exposure of TA, leading concomitantly to the induction of GPx and CAT activity, further studies are needed to clarify the autoxidation properties of TA.

Total SOD and GPx activity was caused by two isoenzymes (Cu/Zn SOD and Mn SOD) and five GPx isoenzymes, respectively. SOD-1 (Cu/Zn SOD) comprises approximately 90% of total SOD activity in a eukaryotic cell (Liu et al., 2004). Of the five isoenzyme, GPx-1 is the major enzyme responsible for removing H₂O₂ (Weydert and Cullen, 2010). We further examined the expression of SOD-1 and GPx-1 in western blot assay. Consistent with the results of enzymatic activities, a significant up-regulated expression of GPx-1 was observed in livers from TA-treated voles, while no significant changes could be detected in the SOD-1 level. In the present study, no depletion in the activities of these antioxidant enzymes were observed, which might be owed to a decreased radical production and the enhanced ability to defend against oxidative stress.

Conclusions

In summary, the present study showed that the growth of Brandt's voles was not

influenced after exposure to 3% dietary TA for five weeks. Dietary TA had an enhancing effect on the average daily food intake, induced the secretion of Pro- and His-containing proteins in saliva and increased their excretion in feces. Consumption of TA also led to the significantly changed expression of two hepatic genes, up-regulated Trf and down-regulated HMGCR, which were responsible for the absorption of iron and homeostasis of lipid metabolism, respectively. A concomitant increase in the hepatic activities of anti-peroxidative enzymes, CAT and GPx, demonstrated that TA contributed to strengthen the potential of antioxidant defense and was beneficial for the suppression of oxidative stress. Our results give direct evidences that more than one adaptive strategies exist in the Brandt's vole which help them to overcome or mitigate the negative effects of dietary TA.

Acknowledgments

This work was financially supported by the National Basic Research Program ("973" Program) of China (2007CB109102), National Science Foundation of China (31470017), and a project funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD). We thank Dr. Zining Wang in MAB and Traits Chromatin Inc., Lubbock, T.X., USA for his valuable advice on the whole manuscript.

References

1. Aebi, H., 1984. Catalase *in vitro*. Methods Enzymol. 105, 121-126.
2. Afsana, K., Shiga, K., Ishizuka, S., Hara, H., 2004. Reducing effect of ingesting tannic acid on the absorption of iron, but not of zinc, copper and

463 manganese by rats. *Biosci. Biotechnol. Biochem.* 68, 584-592.

464 3. Andrade, R.G.Jr., Dalvi, L.T., Silva, J.M.Jr., Lopes, G.K., Alonso, A.,
465 Hermes-Lima M., 2005. The antioxidant effect of tannic acid on the in vitro
466 copper-mediated formation of free radicals. *Arch. Biochem. Biophys.* 437, 1-9.

467 4. Andrews, M., Briones, L., Jaramillo, A., Pizarro, F., Arredondo, M., 2014. Effect
468 of calcium, tannic acid, phytic acid and pectin over iron uptake in an in vitro
469 Caco-2 cell model. *Biol. Trace Elem. Res.* 158, 122-127.

470 5. Bhat, T.K., Kannan, A., Singh, B., Sharma, O.P., 2013. Value addition of feed and
471 fodder by alleviating the antinutritional effects of tannins. *Agric. Res.* 2,189-206

472 6. Bouki, E., Dimitriadis, V.K., Kaloyianni, M., Dailianis, S., 2013. Antioxidant and
473 pro-oxidant challenge of tannic acid in mussel hemocytes exposed to cadmium.
474 *Mar. Environ. Res.* 85, 13-20.

475 7. Chen, S.C., Chung, K.T., 2000. Mutagenicity and antimutagenicity studies of
476 tannic acid and its related compounds. *Food Chem. Toxicol.* 38, 1-5.

477 8. Chung, K.T., Wong, T.Y., Wei, C.I., Huang, Y.W., Lin, Y., 1998. Tannins and
478 human health: a review. *Crit. Rev. Food Sci.* 38, 421-464.

479 9. Fan, H., Wu, D., Tian, W., Ma, X., 2013. Inhibitory effects of tannic acid on fatty
480 acid synthase and 3T3-L1 preadipocyte. *Biochim. Biophys. Acta.* 1831,
481 1260-1266.

482 10. Faria, A., Monteiro, R., Mateus, N., Azevedo, I., Calhau, C., 2007. Effect of
483 pomegranate (*Punica granatum*) juice intake on hepatic oxidative stress. *Eur. J.*
484 *Nutr.* 46, 271-278.

- 485 11. Ferguson, L.R., 2001. Role of plant polyphenols in genomic stability. *Mutat. Res.*
486 475, 89-111.
- 487 12. Gil-Longo, J., González-Vázquez, C., 2010. Vascular pro-oxidant effects
488 secondary to the autoxidation of gallic acid in rat aorta. *J. Nutr. Biochem.* 21,
489 304-309.
- 490 13. Gkouvatsos, K., Papanikolaou, G, Pantopoulos, K., 2012. Regulation of iron
491 transport and the role of transferrin. *Biochim. Biophys. Acta.* 1820, 188-202.
- 492 14. Gomme, P.T., McCann, K.B., Bertolini, J., 2005. Transferrin: structure, function
493 and potential therapeutic actions. *Drug Discov. Today.* 10, 267-273.
- 494 15. Gülçin, İ., Huyut, Z., Elmastaş, M., Aboul-Enein, H.Y., 2010. Radical scavenging
495 and antioxidant activity of tannic acid. *Arab. J. Chem.* 3, 43-53.
- 496 16. Hafeman, D.G., Sunde, R.A., Hoekstra, W.G., 1974. Effect of dietary selenium on
497 erythrocyte and liver glutathione peroxidase in the rat. *J. Nutr.* 104, 580-587.
- 498 17. Hayashi, A., Wada, Y., Suzuki, T., Shimizu, A., 1993. Studies on familial
499 hypotransferrinemia: unique clinical course and molecular pathology. *Am. J.*
500 *Hum. Genet.* 53, 201-213.
- 501 18. Hurrell, R., Egli, I., 2010. Iron bioavailability and dietary reference values. *Am. J.*
502 *Clin. Nutr.* 91, 1461S-1467S.
- 503 19. Hsu, C.L., Yen, G.C., 2007. Effect of gallic acid on high fat diet-induced
504 dyslipidaemia, hepatosteatosis and oxidative stress in rats. *Brit. J. Nutr.* 98,
505 727-735.
- 506 20. Innes, D.G.L., Millar, J.S., 1981. Body weight, litter size, and energetics of

507 reproduction in *Clethrionomys gapperi* and *Microtus pennsylvanicus*. Can. J. Zool.
508 59, 785-789.

509 21. Jaramillo, Á., Briones, L., Andrews, M., Arredondo, M., Olivares, M., Brito, A.,
510 Pizarro, F., 2015. Effect of phytic acid, tannic acid and pectin on fasting
511 iron bioavailability both in the presence and absence of calcium. J. Trace Elem.
512 Med. Bio. 30, 112-117.

513 22. Jin, F., Frohman, C., Thannhauser, T.W., Welch, R.M., Glahn, R.P., 2009. Effects
514 of ascorbic acid, phytic acid and tannic acid on iron bioavailability from
515 reconstituted ferritin measured by an in vitro digestion-Caco-2 cell model. Brit. J.
516 Nutr. 101, 972-981.

517 23. Kalgaonkar, S., Lönnerdal, B., 2008. Effects of dietary factors on iron uptake from
518 ferritin by Caco-2 cells. J. Nutr. Biochem. 19, 33-39.

519 24. Kralova, A., Svetlikova, M., Madar, J., Ulcova-Galova, Z., Bukovsky, A.,
520 Peknicova, J., 2008. Differential transferrin expression in placentae from normal
521 and abnormal pregnancies: a pilot study. Reprod. Biol. Endocrin. 6, 27.

522 25. Lavin, S.R., Chen, Z., Abrams, S.A., 2010. Effect of tannic acid on iron
523 absorption in straw-colored fruit bats (*Eidolon helvum*). Zoo Biol. 29, 335-343.

524 26. Lee, S.H., Shinde, P.L., Choi, J.Y., Kwon, I.K., Lee, J.K., Pak, S.I., Cho, W.T.,
525 Chae, B.J., 2010. Effects of tannic acid supplementation on growth performance,
526 blood hematology, iron status and faecal microflora in weanling pigs. Livest. Sci.
527 131, 281-286.

528 27. Li, J.N., Liu, J.K., Tao, S.L., 2007. Effects of hunger and tannic acid on food

intake and foraging behavior in *Microtus fortis*. Acta Ecologica Sinica
27, 4478-4484.

28. Lindroth, R.L., Batzli, G.O., 1984. Plant phenolics as chemical defenses: Effects
of natural phenolics on survival and growth of prairie voles (*Microtus
ochrogaster*). J. Chem. Ecol. 10, 229-244.

29. Li, Q., Sun, R., Huang, C., Wang, Z., Liu, X., Hou, J., Liu, J., Cai, L., Li, N.,
Zhang, S., Wang, Y., 2001. Cold adaptive thermogenesis in small mammals from
different geographical zones of China. Comp. Biochem. Physiol. A Mol. Integr.
Physiol. 129, 949-961.

30. Liu, J., Hinkhouse, M.M., Sun, W., Weydert, C.J., Ritchie, J.M., Oberley, L.W.,
Cullen, J.J., 2004. Redox regulation of pancreatic cancer cell growth:
role of glutathione peroxidase in the suppression of the malignant phenotype.
Hum. Gene Ther. 15, 239-250.

31. Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data
using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods. 25, 402-408.

32. Li, X.S., Wang, D.H., 2005. Regulation of body weight and thermogenesis
in seasonally acclimatized Brandt's voles (*Microtus brandti*). Horm. Behav. 48,
321-328.

33. Li, Y.G., Yan, Z.C., Wang, D.H., 2010. Physiological and biochemical basis of
basal metabolic rates in Brandt's voles (*Lasiopodomys brandtii*) and Mongolian
gerbils (*Meriones unguiculatus*). Comp. Biochem. Physiol. A Mol. Integr. Physiol.
157, 204-211.

- 551 34. Mansoori, B., Acamovic, T., 2007. The effect of tannic acid on the excretion of
552 endogenous methionine, histidine and lysine with broilers. Anim. Feed Sci. Tech.
553 134, 198-210.
- 554 35. Maqsood, S., Benjakul, S., Balange, A.K., 2012. Effect of tannic acid and kiam
555 wood extract on lipid oxidation and textural properties of fish emulsion sausages
556 during refrigerated storage. Food Chem. 130, 408-416.
- 557 36. Meyer, M.W., Richardson, C., 1993. The effects of chronic tannic acid intake on
558 prairie vole (*Microtus ochrogaster*) reproduction. J. Chem. Ecol. 19, 1577-1585.
- 559 37. Muenzer, J., Bildstein, C., Gleason, M., Carlson, D.M., 1979. Properties of
560 proline-rich proteins from parotid glands of isoproterenol-treated rats. J. Biol.
561 Chem. 254, 5629-5634.
- 562 38. Nepka, C., Asproдини, E., Kouretas, D., 1999. Tannins, xenobiotic metabolism and
563 cancer chemoprevention in experimental animals. Eur. J. Drug Metab. Ph. 24,
564 183-189.
- 565 39. Park, S.Y., Bok, S.H., Jeon, S.M., Park, Y.B., Lee, S.J., Jeong, T.S., Choi, M.S.,
566 2002. Effect of rutin and tannic acid supplements on cholesterol metabolism in
567 rats. Nutr. Res. 22, 283-295.
- 568 40. Pattanayak, S. P., Mazumder, P. M., 2011. Therapeutic potential of *Dendrophthoe*
569 *falcata* (L.f) Ettingsh on 7,12-dimethylbenz (a) anthracene-induced mammary
570 tumorigenesis in female rats: effect on antioxidant system, lipid peroxidation, and
571 hepatic marker enzymes. Comp. Clin. Pathol. 20, 381-392.
- 572 41. Pei, Y.X., Wang, D.H., Hume I., 2001. Selective digesta retention and coprophagy

573 in Brandt's vole (*Microtus brandti*). J. Comp. Physiol. B. 171, 457-464.

574 42. Robbins, C.T., Hagerman, A.E., Austin, P.J., McArthur, C., Hanley, T.A., 1991.

575 Variation in mammalian physiological responses to a condensed tannin and its

576 ecological implications. J. Mammal. 72, 480-486.

577 43. Shimada, T., 2006. Salivary proteins as a defense against dietary tannins. J. Chem.

578 Ecol. 32, 1149-63.

579 44. Shimada, T., Saitoh, T., 2003. Negative effects of acorns on the wood mouse

580 *Apodemus speciosus*. Popul. Ecol. 45, 7-17.

581 45. Skopec, M.M., Hagerman, A.E., Karasov, W.H., 2004. Do salivary proline-rich

582 proteins counteract dietary hydrolyzable tannin in laboratory Rats? J. Chem. Ecol.

583 30, 1679-1692.

584 46. Song, Z.G., Wang, D.H., 2006. Basal metabolic rate and organ size in Brandt's

585 voles (*Lasiopodomys brandtii*): Effects of photoperiod, temperature and diet

586 quality. Physiol. Behav. 89, 704-710.

587 47. Sri Kumar, R., Parthasarathy, N.J., Manikandan, S., Narayanan, G.S., Sheeladevi,

588 R. 2006. Effect of Triphala on oxidative stress and on cell-mediated immune

589 response against noise stress in rats. Mol. Cell. Biochem. 283, 67-74.

590 48. Tikoo, K., Sane, M.S., Gupta, C., 2011. Tannic acid ameliorates

591 doxorubicin-induced cardiotoxicity and potentiates its anti-cancer activity:

592 potential role of tannins in cancer chemotherapy. Toxicol. Appl. Pharm. 251,

593 191-200.

594 49. Wallander, M.L., Leibold, E.A., Eisenstein, R.S., 2006. Molecular control of

vertebrate iron homeostasis by iron regulatory proteins. Biochim. Biophys. Acta .1763, 668-689.

50. Weydert, C.J., Cullen, J.J., 2010. Measurement of superoxide dismutase, catalase and glutathione peroxidase in cultured cells and tissue. Nat. Protoc. 5, 51-66.

51. Wu, L.T., Chu, C.C., Chung, J.G., Chen, C.H., Hsu, L.S., Liu, J.K., Chen, S.C., 2004. Effects of tannic acid and its related compounds on food mutagens or hydrogen peroxide-induced DNA strands breaks in human lymphocytes. Mutat. Res. 556, 75-82.

52. Yugarani, T., Tan, B.K., Teh, M., Das, N.P., 1992. Effects of polyphenolic natural products on the lipid profiles of rats fed high fat diets. Lipids. 27,181-186.

53. Zhang, L., Fang, G., Zheng, L., Chen, Z., Liu, X., 2013. Hypocholesterolemic effect of capsaicinoids in rats fed diets with or without cholesterol. J. Agr. Food Chem. 61, 4287-4293.

54. Zhang, Q., Lin, Y., Zhang, X.Y., Wang, D.H., 2015. Cold exposure inhibits hypothalamic Kiss-1 gene expression, serum leptin concentration, and delays reproductive development in male Brandt's vole (*Lasiopodomys brandtii*). Int. J. Biometeorol. 59, 679-691.

55. Zhang, X.Y., Jing, B.B., Wang, D.H., 2009. Cold exposure does not decrease serum leptin concentration, but increases energy intake and thermogenic capacity in pregnant Brandt's voles (*Lasiopodomys brandtii*). J. Zool. 112, 206-216.

56. Zhang, X.Y., Wang, D.H., 2006. Energy metabolism, thermogenesis and body mass regulation in Brandt's voles (*Lasiopodomys brandtii*) during cold

617 acclimation and rewarming. Horm. Behav. 50, 61-69.

618 57. Zhang, X.Y., Wang, D.H., 2007. Thermogenesis, food intake and serum leptin in
619 cold-exposed lactating Brandt's voles (*Lasiopodomys brandtii*). J. Exp. Biol. 210,
620 512-521.

621 58. Zhang Z.B., Wang, Z.W., 1998. Ecology and management of rodent pests in
622 agriculture. Ocean Press, Beijing, pp209-220.

623 59. Zhao, Z.J., Wang, D.H., 2007. Effects of diet quality on energy budgets and
624 thermogenesis in Brandt's voles. Comp. Biochem. Physiol. A Mol. Integr. Physiol.
625 148, 168-177.

626

Table 1 Nucleotide sequences of primers used for amplification.

Gene	Primer	Sequence (5' to 3')	Size of amplicon (bp)
<i>HMGCR</i>	HMGCR-up	TTTGTGGCCTCCCAY*CCN*TGGGA	2282
	HMGCR-down	CCCACATTCTGTGCTGCR*TCY*TG	
	HMGCR-qPCRup	CTGGATCGAAGGAAGAGGCAA	182
	HMGCR-qPCRdown	TGACGATGTTGGCTGCATGG	
<i>Trf</i>	Trf-up	CTTACTTCATATTTCA Y*GCCTGGW*GA	427
	Trf-down	GTGGTAGGTTTGGTGGTN*GGN*GCN*GG	
	Trf-qPCRup	ATTCAGTCAAGGCTGTGCTC	238
	Trf-qPCRdown	TCAAAGTCTGTCAGCTTCGGA	
β -actin	β -actin- qPCRup	TTGTGCGTGACATCAAAGAG	199
	β -actin- qPCRdown	ATGCCAGAAGATTCCATACC	

627 * Y=C or T; R=A or G; W= A or T; N=A, T, C or G.

Table 2 Effects of dietary TA on body weight, food intake, organ weights, organ parameters and total Pro and His contents in saliva and feces samples of Brandt's voles.

	Control group	TA-treated group
Body weight (g)	34.61 ± 1.36	35.12 ± 0.77
Food intake (g/day) *	13.09 ± 0.27	14.88 ± 0.34
Organ weights (g)		
Liver	1.23 ± 0.06	1.29 ± 0.05
Heart	0.18 ± 0.01	0.19 ± 0.01
Kidney	0.37 ± 0.01	0.37 ± 0.01
Organ parameters (%)		
Liver	3.63 ± 0.13	3.67 ± 0.15
Heart	0.54 ± 0.02	0.55 ± 0.02
Kidney	1.08 ± 0.04	1.07 ± 0.03
Content of total Pro		
Saliva (µg/µL) *	0.54 ± 0.03	0.92 ± 0.05
Feces (µg/g) *	1105.37 ± 80.13	1968.67 ± 79.33
Content of total His		
Saliva (µg/µL) *	0.24 ± 0.01	0.40 ± 0.01
Feces (µg/g) *	605.98 ± 51.10	956.68 ± 64.13

* significantly different ($P < 0.01$) between two groups.

639 **Fig. 1.** The profile of salivary proteins. Saliva samples were separated on 10%
640 SDS-PAGE. A total of 25 μ L saliva was loaded on each lane. Lane1-4, saliva samples
641 from TA-treated group; Lane 5-8, saliva samples from the control group.
642

Fig. 2. Hepatic expression of *HMGCRC* gene and *Trf* gene in Brandt's voles. Values were expressed as mean $\pm$ SD of six animals. The ** symbol represented statistical significance at $P < 0.01$.

Fig. 3. Western blot analyses of hepatic HMGCR, Trf, SOD-1, and GPx-1. β -actin was used as a lane-loading control. Lysates were loaded on 10% acrylamide gels (SDS, reducing conditions). The intensities of relative band were determined by densitometry and normalized to β -actin.

Fig. 4. Activities of enzymatic antioxidants in the liver tissue of Brandt's voles. Enzymatic activities of total GPx, CAT, and total SOD were shown by bar graphs on the bottom. Values for total GPx activity were shown on the left Y-axis as U/g protein. Values for the CAT and total SOD activities were shown on the right Y-axis as U/mg protein. The symbol * indicated significant difference at $P < 0.05$. Data from 10 individual voles were presented as Mean $\pm$ SD.