Pol. J. Environ. Stud. Vol. 30, No. 5 (2021), 3919-3926
DOI, *10.15244/pjoes/132792* ONLINE PUBLICATION DATE: 2021-06-11

Review

Effects of Sulfur Fertilization on Antioxidant Capacity of Wumeng Semi-Fine Wool Sheep in the Wumeng Prairie

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Received: 16 November 2020
Accepted: 25 January 2021

Abstract

To assess the impact of sulfur(S) fertilization on antioxidant capacity of grazing Wumeng semi-fine wool sheep in copper(Cu) polluted meadow, and explore the control methods of Cu pollution in natural pasture, fertilizer treatment and grazing experiments were carried out in Weining County of the Wumeng Prairie, Guizhou Province, Southwest China. 24 ha Cu-polluted meadows were fenced, and randomly divided into four groups (3 replications/group, 2 ha/replication): 1) control group, no fertilizer; 2) the tested groups, applied ammonium sulfate [$(NH4)_2SO_4$] (300, 400, and 500 kg/ha for group I, group II, and group III, respectively). A total of 72 healthy Wumeng semi-fine wool sheep (aged one year, 32.8±1.3 kg) were used in this study. All animals were randomly divided into 4 groups (3 replications/group, 6 sheep/replication) and assigned to the tested pastures. The grazing experiment lasted for 60 days. The results showed that the contents of N, Mn, Zn, and S in herbage in fertilized pastures were markedly higher than those in control group (P<0.01). The contents of Cu, Fe, and Se in herbage in fertilized pastures were significantly lower than those in control group (P<0.01). There were no extreme differences among the fertilized pastures (P>0.05). The contents of Mn, Zn, and S in serum and liver in the tested sheep were significantly higher than those in control animals (P<0.01). The contents of Cu, Fe, and Se in serum and liver in the tested sheep were extremely lower than those in control animals (P<0.01). The levels of Hb, RBC, and PCV in blood in the tested sheep, and the activities of serum SOD, GSH-Px, T-AOC, CAT, and Cp in the tested sheep were extremely higher than those in control sheep (P<0.01). The contents of serum MDA in the tested sheep were significantly lower than those in control animals (P<0.01). There were no great differences among the tested groups in physiological and biochemical parameters (P>0.05). The current results indicated that fertilization of

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(NH4)2SO4 not only markedly influenced the mineral contents of herbage, and samples of blood and liver, but also extremely improved antioxidant capacity in grazing animals from fertilized pastures.

Keywords: Wumeng semi-fine wool sheep, copper polluted meadow, fertilization treatment, mineral content, antioxidant capacity

Introduction

The Wumeng Prairie is located in the northwest of Guizhou Province, Southwest China. It has extremely rich mineral resources. Over the last 10 years, due to rapid development of metallurgical industries, as a result, a large number of heavy metals had been produced in the Wumeng Prairie [1]. The Cu contents in water, soil, and forage have increased extremely in Weining County of the Wumeng Prairie, Guizhou Province, Southwest China. Cu elements are not easy to decompose in natural pasture, so the polluted natural ranges are very difficult to be repaired [1]. Cu pollution not only seriously damaged animal husbandry, but also greatly threatened human health through the food chain [2, 3].

The Wumeng Prairie is a key pastureland for Wumeng semi-fine wool sheep. These native sheep were also vital to the production system in the Wumeng Prairie, and mainly distributed in the high altitude pasture in Weining County of the Wumeng Prairie, Southwest China [4, 5]. Wumeng semi-fine wool sheep have high growth performance, mild temperament and strong adaptability to alpine pastoral ranges [6, 7]. However, their natural habitats in the Wumeng Prairie have been seriously polluted by heavy metals. Heavy metal pollution leads to the destruction of soil structure and loss of soil fertility, which not only affects the yield and quality of forages but also badly damages animal and human health [8]. The sheep industry is also grievously hindered in the Wumeng Prairie, Southwest China. Therefore, it is urgent to explore the effective control measures of Cu pollution in the Wumeng Prairie. To reduce the intake of heavy metals in human beings, the most effective way is to keep heavy metals out of plants and animals. The previous study found that sulfur in feed is converted into sulfide in the rumen, leading to precipitation of Cu sulfide and hindering the absorption and utilization of Cu [8]. The interference means of fertilization and grazing might change the biodiversity of grazing land and foraging strategies in grazing animal, as a result, pollution elements into the animal body is reduced [9]. Adjusting the soil pH and increasing contents of organic matter can much change the activities of heavy metals in soil, and the bioavailability of heavy metals is also reduced. However, it will take a long time to control heavy metal pollution.

The objective of this study was to determine the effects of S fertilization and grazing measures on antioxidant capacity of Wumeng semi-fine wool sheep, and explore the new methods and technologies for the management of Cu-polluted pastures.

Materials and Methods

Purchase of Ammonium Sulfate

Ammonium sulfate [$(NH_4)_2SO_4$] was purchased from Luxi Chemical Industry Group Co. Ltd., with purity of no less than 99%.

Experimental Design

The Cu-polluted pastures were located in Weining County of the Wumeng Prairie, Guizhou Province. Before the test, the mineral contents in soil, herbage, samples of serum and liver were analyzed. 24 ha Cu-polluted meadow was selected for this study, and randomly divided into four groups (3 replications/group, 2 ha/replication): 1) control group, with no fertilizer; 2) the tested groups, applied $(NH_4)_2SO_4$ (300, 400, and 500 kg/ha for group I, group II, and group III, respectively). 72 healthy Wumeng semi-fine wool sheep (aged one year, 32.8±1.3kg) were used in this study. All animals were randomly divided into four groups (3 replications/group, 6 sheep/replication) and assigned to the fertilized pastures. The grazing experiment lasted for 60 days.

Feeding Management

The experimental houses were all semi-open type, and thoroughly cleaned and disinfected during the trial period. The houses were disinfected twice/a month (2% caustic soda or 0.5% povidone iodine, alternate use). Animals were grazing in the daytime and fed concentrate supplementation at 18:00 once a day. Water provided ad libitum. The health status of sheep was recorded every day.

Sample Collection and Analysis

Soil and herbage samples were collected and prepared according to Huo et al. [10]. On day 60 of the experiment, after fasting for 12 hours, blood samples were collected from the jugular vein into vacuum blood collection tubes with EDTA-K_2 anticoagulant, and stored at 4ºC for up 4 hours until assay of hematology examination. Serum samples were collected using vacuum blood collection tubes with 1% heparin sodium, separated by centrifuge at 3 000×g for 15 min,

Table 1. The mineral contents in soil and herbage in Cu-polluted pasture.

Items	Soil	Herbage
Mn(mg/kg)	202.24±27.17	61.54±10.20
Zn(mg/kg)	52.12±11.08	84.62±12.21
Co(mg/kg)	6.48±1.28	1.48±0.35
Cu(mg/kg)	76.87±4.93a	37.22±2.61a
Fe(mg/kg)	15008.42±275.15	318.63±20.51
Mo(mg/kg)	1.88±0.35	1.22±0.15
Se(mg/kg)	0.12±0.02	0.12±0.01
S(%)	0.18±0.04	0.25±0.02

Note: Mn = manganese, Zn = zinc, Co = cobalt, Cu = copper, Fe = iron, Mo = molybdenum, Se = selenium, S = sulphur. Different lowercase letters in the same trade indicates significant difference at $P<0.01$ level.

and stored in an EP tube at -80ºC for analysis of trace elements and enzyme activities. The living liver samples of Wumeng semi-fine wool sheep were collected by skilled technicians through liver puncture, fixed in the sample bottle, then stored at -80ºC until analyzed.

Soil samples were naturally dried at 20-25ºC and crushed, then passed through a 0.075-mm sieve used to remove silver sand. Herbage and blood samples were heated and digested by microwave radiation in sealed pot, then determined by computer. The levels of manganese (Mn), zinc (Zn), cobalt (Co), copper (Cu), iron (Fe), molybdenum (Mo), and selenium (Se) were performed by Perkin Elmer, Norwalk, USA. Nitrogen (N) element was analyzed by silver nitrate titration [11], and S element was analyzed by barium sulfate gravimetric method [12].

Levels of hemoglobin (Hb), erythrocyte count (RBC), packed cell volume (PCV), and white blood cell count (WBC) were determined by automatic blood cell analyzer (SF-3000, Sysmex-Toa Medical Electronics, Kobe, Japan). Serum superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), total antioxidant capacity (T-AOC), catalase (CAT), malondialdehyde (MDA), and ceruloplasmin(Cp) were determined using commercial test kits (Nanjing Jiancheng Bio-Engineering Institute, China).

Statistical Analyses

Experimental data were analyzed by using the statistical package (SPSS, version 23.0, Inc., Chicago, Illinois, USA), and presented in the form of mean±standard deviation (SD). One way ANOVA program was used to analyze the data and Duncan method was used for multiple comparisons. A probability level of $P<0.01$ were considered to be extremely significant. $P>0.05$ indicates no significant difference.

Results

The Mineral Contents in Soil and Herbage

As shown in Table 1, the contents of Cu in soil and forage were 76.87 and 37.22 mg/kg, respectively, far exceeding the normal levels [13].

Effects of S Fertilization on Mineral Contents in Herbage

As shown in Table 2, compared to control group, the contents of N, Mn, Zn, and S in the tested groups extremely increased ($P<0.01$), and the contents of Cu, Fe, and Se in the tested groups significantly decreased ($P<0.01$), but there were no extreme differences among the tested groups ($P>0.05$).

Table 2. Effects of S fertilization on mineral contents in herbage in Cu-polluted pasture.

Items	Control	Group I	Group II	Group III
N(%)	2.29±0.20b	3.52±0.22a	3.58±0.21a	3.62±0.23a
Mn(mg/kg)	61.52±10.21b	81.72±14.90a	85.33±10.61a	86.82±16.60a
Zn(mg/kg)	84.64±12.20b	104.64±12.10a	114.62±10.93a	115.83±16.21a
Co(mg/kg)	1.48±0.35	1.37±0.12	1.43±0.11	1.52±0.18
Cu(mg/kg)	17.26±2.63a	12.92±2.10b	13.02±2.20b	13.74±2.01b
Fe(mg/kg)	318.64±20.51a	285.43±11.31b	289.24±10.11b	290.52±20.30b
Mo(mg/kg)	1.22±0.15	1.21±0.10	1.29±0.13	1.24±0.12
Se(mg/kg)	0.12±0.01a	0.07±0.02b	0.08±0.01b	0.08±0.02b
S(%)	0.25±0.02b	0.32±0.05a	0.31±0.07a	0.33±0.03a

Note:N = nitrogen, Mn = manganese, Zn = zinc, Co = cobalt, Cu = copper, Fe = iron, Mo = molybdenum, Se = selenium, S = sulphur. Different lowercase letters in the same trade indicates significant difference at $P<0.01$ level.

Effects of S Fertilization on Mineral Contents in Serum

As shown in Table 3, compared to control group, the contents of serum Mn, Zn, and S in the tested groups extremely increased (*P*<0.01), and the contents of serum Cu, Fe, and Se significantly decreased (*P*<0.01), but there were no extreme difference among the tested groups (*P*>0.05).

Effects of S Fertilization on Mineral Elements in Liver

As shown in Table 4, compared to control group, the contents of liver Mn, Zn, and S in the tested groups extremely increased (*P*<0.01), and the contents of liver Cu, Fe, and Se significantly decreased (*P*<0.01), but there were no extreme difference among the tested groups (*P*>0.05).

Table 3. Effects of S fertilization on mineral contents in serum of grazing sheep.

Items	Control	Group I	Group II	Group III
Mn(mg/kg)	0.52±0.04b	0.77±0.11a	0.75±0.10a	0.73±0.12a
Zn(mg/kg)	7.40±0.13b	8.65±0.12a	8.68±0.10a	8.67±0.10a
Co(mg/kg)	0.67±0.12	0.66±0.18	0.71±0.20	0.70±0.15
Cu(mg/kg)	6.56±0.55a	3.38±0.55b	3.49±0.42b	3.46±0.33b
Fe(mg/kg)	374.91±38.22a	314.84±40.21b	310.83±55.20b	315.74±38.21b
Mo(mg/kg)	0.07±0.02	0.08±0.01	0.09±0.03	0.07±0.02
Se(mg/kg)	0.16±0.03a	0. 10±0.03b	0.10±0.02b	0.13±0.02b
S(%)	0.08±0.02b	0.12±0.03a	0.13±0.02a	0.13±0.02a

Note: Mn = manganese, Zn = zinc, Co = cobalt, Cu = copper, Fe = iron, Mo = molybdenum, Se = selenium, S = sulphur.
Different lowercase letters in the same trade indicates significant difference at *P*<0.01 level.

Table 4. Effects of S fertilization on mineral contents in liver of grazing sheep.

Items	Control	Group I	Group II	Group III
Mn(mg/kg)	4.11±1.36b	5.15±1.20a	5.21±1.35a	5.28±1.33a
Zn(mg/kg)	76.45±11.82b	83.63±16.21a	87.42±18.50a	81.84±15.70a
Co(mg/kg)	7.31±1.09	7.34±1.21	7.31±1.30	7.46±1.08
Cu(mg/kg)	1868.26±40.11a	1078.42±26.15b	1001.21±28.15b	1039.22±28.10b
Fe(mg/kg)	351.08±37.12a	305.12±18.04b	308.07±13.05b	304.34±19.06b
Mo(mg/kg)	2.98±0.30	2.82±0.23	2.79±0.22	2.81±0.23
Se(mg/kg)	1.23±0.06a	0.86±0.04b	0.88±0.03b	0.90±0.02b
S(%)	0.68±0.02b	1.21±0.03a	1.28±0.02a	1.30±0.02a

Note: Mn = manganese, Zn = zinc, Co = cobalt, Cu = copper, Fe = iron, Mo = molybdenum, Se = selenium, S = sulphur.
Different lowercase letters in the same trade indicates significant difference at *P*<0.01 level.

Table 5. Effects of S fertilization on physiological indexes of grazing sheep in Cu-polluted pasture.

Items	Control	Group I	Group II	Group III
Hb(g L^{-1})	83.12±12.50b	103.00±11.62a	103.24±11.90a	102.32±13.81a
RBC(10^{12} L^{-1})	6.83±1.11b	9.53±1.81a	9.83±1.52a	9.72±1.30a
PCV (%)	30.52±2.30b	32.42±2.13a	32.51±1.73a	32.03±2.50a
WBC(10^{9} L^{-1})	10.52±1.50	9.61±1.32	9.94±1.20	10.43±1.31

Note: Hb = hemoglobin, RBC = erythrocyte count, PCV = packcd cell volume, WBC = white blood cell count.
Different lowercase letters in the same trade indicates significant difference at *P*<0.01 level.

Table 6. Effects of S fertilization on antioxidant capacity of grazing sheep in Cu-polluted pasture.

Items	Control	Group I	Group II	Group III
SOD (IU/mL)	33.95±2.92b	58.53±4.62a	55.73±3.82a	57.22±4.30a
GSH-Px (IU/mL)	24.14±2.90b	29.64±2.72a	29.54±4.13a	29.84±3.22a
T-AOC (IU/mL)	6.85±1.66b	7.96±0.85a	7.88±0.96a	7.93±0.63a
CAT (IU/mL)	0.73±0.15b	1.04±0.12a	1.05±0.11a	1.08±0.12a
MDA (nmol/mL)	35.34±3.21a	31.23±2.62b	31.03±3.10b	31.43±3.21b
Cp (BU/dL)	8.82±1.18b	10.39±1.12a	10.93±0.95a	11.06±1.15a

Note: SOD = superoxide dismutase, GSH-Px = glutathione peroxide, T-AOC = total antioxidant capacity, CAT = catalase, MDA = malondialdehyde, Cp = ceruloplasmin. Different lowercase letters in the same trade indicates significant difference at $P<0.01$ level.

Effects of S Fertilization on Physiological Indexes

As shown in Table 5, compared to control group, the levels of Hb, RBC, and PCV in the tested groups extremely increased ($P<0.01$), but there were no extreme difference among the tested groups ($P>0.05$).

Effects of S Fertilization on Antioxidant Capacity

As shown in Table 6, compared to control group, the activities of serum SOD, GSH-Px, T-AOC, CAT, and Cp extremely increased in the tested groups ($P<0.01$), and the contents of serum MDA significantly decreased($P<0.01$), but there were no extreme difference among the tested groups ($P>0.05$).

Discussion

Effects of S Fertilization on Mineral Elements in Herbage

Mineral elements are key nutrients for animals and humans [5, 14]. Too high contents of mineral elements in soil and herbage will cause mineral poisoning in animals [15]. Therefore, the study on the characteristics of mineral elements in soil, herbage, and livestock can not only clarify the distribution law of minerals in pasture, but also help to understand status of nutrition in grazing animals [16, 17].

N and S are important nutrients in plants. The previous study found that application of $(NH_4)_2SO_4$ can extremely increase the contents of N and S in herbage [18]. The Cu content of forage from the tested pasture in this study was 76.87 mg/kg, higher than the soil environmental quality risk control standard of soil contamination of agricultural land in China [13]. The ion concentrations in soil were serious influenced by $(NH_4)_2SO_4$ treatment. The physical and chemical properties of soil had changed greatly. The status of the mineral nutrition in soil was also altered, as a result, the diversity of plant community and the mineral contents in herbage was greatly affected in pasture [19]. In this study, the contents of N, Zn, Mn, and S in fertilized pastures extremely increased and the contents of Cu, Fe, and Se greatly reduced. These results were different from the study of Shen XY et al. [18]. This might be related to the mineral contents in soil before the experiments.

Generally, $(NH_4)_2SO_4$ is dissolved in water solution in soil and ionized into ammonium ion and sulfate ion. Because the amount of ammonium ions selectively absorbed by plants is larger than those of sulfate ions, the residual sulfate ions in soil combine with hydrogen ions and make soil acid [20], which is associated with an increase in Zn and Mn uptake in forage [12]. Therefore, in this study, the contents of Zn and Mn in forage in fertilized pastures significantly increased.

S element is an important factor affecting the absorption of Se by plants. The solubility of selenate in soil is higher than that of selenite, often coexists with sulfate and is easy to be absorbed by plants [14]. It was found that there was a competitive antagonism between selenate and sulfate. Selenate and sulfate competed for the same absorption site in plant roots, and the ratio of selenate and sulfate determined their absorption degree [21]. Therefore, the interaction between S and Se was the main reason for the low Se contents in herbage in $(NH_4)_2SO_4$ fertilized pastures [21]. S fertilization accelerated the transfer of Se from soil to plants. The available Se contents in soil inevitably decreased with the growth of plants, then the Se contents in plants reduced. However, the interaction between S and Se was very complex and needed further study.

Effects of S Fertilization on Antioxidant Capacity

There are harmful consequences of extremely excessive mineral nutrients in animals and humans. The number of reported chronic Cu poisoning cases rises steadily in China, due partly to the growing popularity of susceptible breeds, such as Wumeng semi-fine wool sheep [8, 22, 23]. In addition, metallothionein (MT) and metal binding protein (MBP) can be regulated to prevent or reduce its toxicity. Cu plays an important role in enhancing immune function [24, 25]. A large number of oxygen free radicals are produced in the metabolism of the body, which causes denaturation of protein and nucleic acid, degradation of polysaccharide and formation of lipid peroxide [26]. Oxygen free radicals produce hydrogen peroxide under the action of SOD. Hydrogen peroxide is degraded and removed by CAT and GSH-Px, which keeps the organism in a good immune state [27]. Cu is also a component of SOD catalytic center in organism, which is related to SOD catalytic activity [22]. When the Cu content in the body is extremely excessive, it shows certain toxicity. Excessive Cu promotes the oxidative modification of low-density lipoprotein (LDL). The lipid peroxidation produced malondialdehyde and other lipid peroxides, as a result, organism is damaged by the free radicals [28].

The liver is the key organ for storing Cu in animals. High Cu can damage the subcellular structures, such as the nucleus, mitochondria, and serous fluid of liver [29]. If the Cu intake in liver exceeds its tolerance limit, it can inhibit the activities of various enzymes, and causes necrosis of liver cells. This makes the dysfunction of liver excreting Cu, then leads to Cu accumulation. When the Cu concentration in liver is very high, a large amount of Cu is released into blood, then enters into red blood cells in the action of certain inducement. The continuously increasing of intracellular Cu will greatly reduce the concentration of glutathione in red blood cells, increase the brittleness of red blood cells, and cause intravascular hemolysis [30, 31], as a result, hemolytic anemia occurs [31]. The previous study found that S interference with Cu absorption caused Cu deficiency in Wumeng semi-fine wool sheep [31]. In normal conditions, most of Cu in blood exists in the form of ceruloplasmin (Cp). Cp has the function of ferrous oxidase and anti-oxidation. The lack of Cu often leads to the decrease of Cp in blood. Generally, Fe^{3+} in food is reduced to Fe^{2+} in gastrointestinal tract before it can be absorbed by animals. Fe^{2+} enters animal body, combines with carboxyferritin and is changed into Fe^{3+}, then stored in liver. Fe^{3+} in liver can be reduced to Fe^{2+} again and enters into blood. Fe^{2+} in blood is oxidized to Fe^{3+}, then Fe^{3+} combines with β-globulin to form iron transfer protein, which is sent to bone marrow to synthesize Hb. *In vivo* Cp regulates the transformation between liver Fe^{3+} and blood Fe^{2+} [31]. Therefore, the decreased Cp concentration in blood will cause that Fe stored in liver cannot be mobilized. Fe stored in the phagocytes cannot enter blood, as a result, Fe content in blood decreases, and hemolytic anemia occurs in animals [11, 31]. In this study, the contents of blood Cu and Fe in $(NH_4)_2SO_4$ treatment significantly reduced in grazing animals from fertilized pastures, but the Cu contents were still far higher than the healthy level, so the anemia symptoms of grazing sheep were only relieved to some extent.

The antioxidant system is the defense system for scavenging free radicals, comprising non-enzymatic (Cu, Fe, Zn, Se) and enzymatic systems (SOD, GSH-Px, T-AOC, CAT, and other antioxidant enzymes) [32-34]. SOD can catalyze the superoxide anion (O^{2-}) to produce disproportionation reaction, remove superoxide anion, and protect cells from damage [35,36]. GSH-Px and CAT can clear H_2O_2 in the body, so as to protect cells from H_2O_2 damage [28]. As the comprehensive indicator for evaluating levels of antioxidant enzymes and the non-enzymatic system in animal organisms, T-AOC can reflect the compensatory capacity to external stimuli and the metabolism capacity of free radicals [22, 37]. Decreased level of T-AOC cannot keep antioxidant system active, leading to the abundance of lipid peroxides and free radicals. MDA is a lipid peroxide, which can reflect the degree of lipid peroxidation in the body [38]. The reaction product of SOD is the substrate of GSH-Px [39]. The radical scavenging of O^{2-} requires continuous catalysis from SOD, GSH-Px, and CAT [40,41]. Therefore, SOD, GSH-Px, and CAT play a more important role in blocking membrane lipid peroxidation induced by free radicals. In our study, the contents of mineral elements in herbage were extremely affected by application of $(NH_4)_2SO_4$ fertilization. The Cu contents in herbage, serum, and liver of grazing animals were significantly higher than those in control group, the activities of serum SOD, GSH-Px, T-AOC, CAT, and Cp were significantly lower than those in control group. The contents of MDA were extremely higher than those in control group. Although the contents of Mn, Zn, and S in serum and liver extremely increased, and the activities of antioxidant enzymes in serum significantly enhanced, application of S fertilization could not interfere with the Cu metabolism of Wumeng semi-fine wool sheep. Therefore, hemolytic anemia still occurred in grazing animals in Cu-polluted meadows. Application of S fertilization only partly alleviated the Cu toxicity on antioxidant system in grazing Wumeng semi-fine wool sheep.

Conclusion

The Cu contents in herbage were significantly influenced by S fertilization in Cu-polluted pastures, but it cannot completely eliminate damage of the Cu pollution on antioxidant capacity in grazing Wumeng semi-fine wool sheep. It is necessary to further explore the methods to control Cu pollution in natural range lands.

Acknowledgements

We gratefully acknowledge support by the Project of National Key Research and Development Program of China in 13th Five-year Plan (2016YFC0502601) and the National Natural Science Foundation of China (41671041).

Conflict of Interest

The authors declare no conflict of interest.

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