Pol. J. Environ. Stud. Vol. 29, No. 4 (2020), 2917-2924
DOI: *10.15244/pjoes/111881* ONLINE PUBLICATION DATE: 2020-02-14

Original Research

Response of Wumeng Semi-Fine Wool Sheep to Copper-Contaminated Environment

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Received: 2 June 2019
Accepted: 21 August 2019

Abstract

We evaluated the response of Wumeng semi-fine wool sheep to a copper-contaminated environment and found an action plan to solve copper pollution through a grazing experiment and ammonium molybdate supplementary experiment carried out in Weining County of Guizhou Province in China. The content of heavy metal element in soil, herbage, and animal tissues was measured by atomic absorption spectrometry, and the blood physiological and biochemical indicators were determined by animal-specific automatic blood analyzer and automatic biochemical analyzer respectively. The results showed that the copper content in soil and herbage of contaminated pasture was significantly higher than that in control pasture, and the copper content in blood and liver in affected sheep was significantly higher than that in the control group. Hemoglobin (Hb), red blood cell count (RBC), hematocrit (PCV), mean corpuscular volume (MCV) contents and superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSH-Px) activities in affected sheep were significantly lower than those in control, while the activities of ceruloplasmin (CP), glutamic oxaloacetic transaminase (GOT), creatine phosphokinase (CPK) and malondialdehyde (MDA) were significantly higher. There was no significant difference in the level of mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and white blood cell count (WBC) between the affected and control sheep. After supplementation of ammonium molybdate, copper content in blood and liver decreased gradually, and the abnormal blood indexes recovered. At the end of the ammonium molybdate supplementation experiment, Wumeng semi-fine wool sheep in the drug-control group (CK group) showed hemoglobinuria, jaundice, anemia and other symptoms. Conclusion: a copper-contaminated environment seriously affected mineral metabolism and blood physiological and biochemical indicators of Wumeng semi-fine wool sheep, and we can utilize the antagonism of molybdenum and copper in the diet to achieve the goal of harmless utilization of a copper-polluted meadow.

Keywords: Wumeng semi-fine wool sheep, heavy metal, copper-contaminated environment, blood index

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Introduction

Wumeng semi-fine wool sheep, formerly known as Guizhou semi-fine wool sheep, is a variety that was examined and approved in the early 1990s [1]. They were bred by introducing Weining sheep (Tibetan valley type coarse wool sheep) with Corriedale, Merino sheep and Xinjiang fine wool sheep. Hence, it has some excellent characteristics, such as good adaptability, greater disease resistance, great ability to endure roughage, obedient disposition, and easy management, and the demand for forage quality is not high [2]. Compared with Weining sheep, production performance was significantly improved. The average body weights of adult rams and ewes are 61.62±5.38 kg and 52.76±5.93 kg respectively, the average wool yields are 5.86±1.27 kg, and 4.72±1.13 kg respectively, the main fineness of wool is 25.00-29.00 μm, wool yield is 57.72%, and the lambing rate is 119.59% [1, 3]. At present, Wumeng semi-fine wool sheep have been extended to Weining, Hezhang and Shuicheng counties in Guizhou Province, accounting for 62% of sheep in stock. Wumeng semi-fine wool sheep play an important role in the grassland stockbreeding economy of Guizhou and is the main source of local productive life material in the Wumeng mountainous area [4].

Heavy metal pollution refers to the environmental pollution caused by heavy metals or their compounds [5]. Heavy metal pollution was the result of artificial causes such as mining, exhaust and wastewater emissions, sewage irrigation, etc. [6]. Heavy metals in soil are difficult to degrade, and the high accumulation of these metals induces adverse effects on plant growth, which may cause the problem of toxicities in animals depending on plants and forage material [7, 8]. Wumeng mountainous area is the natural habitat of Wumeng semi-fine wool sheep, where forage resources and unique natural conditions for developing grassland animal husbandry abound. However, in recent years, due to the exploitation and utilization of mineral resources, the area has become a heavy metal pollution area in China, with copper one of the main culprits, which seriously affects the development of local animal husbandry [5].

For the purpose of studying the effects of a copper-contaminated environment on Wumeng semi-fine wool sheep, the mineral elements content in soil, herbage, blood and liver, and blood indexes of Wumeng semi-fine wool sheep were detected in this experiment. By adding different levels of ammonium molybdate into the diet to found the suitable treatment methods, which could provide reference for the prevention and treatment of chronic copper poisoning and loss reduction of the sheep-breeding industry.

Materials and Methods

Study Area

The study area is located in Weining County (26°30’-27°25’N, 103°36’-104°45′E), which belongs to the subtropical and warm temperate monsoon climate, with an average altitude of 1550-2200 m, annual average temperature of 11.5-11.8ºC, a frost-free period of 180-257 d, annual average rainfall of 890-1150 mm, and average sunshine duration of 1400-1800 h.

Experimental Design

20 Wumeng semi-fine wool sheep were selected from the pasture of Niupeng town (affected group) and Liangshuigou sheep stud farm (control group), respectively. Mineral elements in soil, herbage, blood and liver were determined, and blood physiological and biochemical indexes were measured. Then, 20 sheep from the control pasture were transferred to an affected pasture and randomly divided into 4 groups for isolation grazing. After 30 days, combined with the test results of Guan [9], 3 groups of sheep were supplemented with ammonium molybdate ($(NH_4)_2Mo_2O_7$) with 100, 200, 300 mg/kg respectively for 60 days (2 every 10 d), and the rest of the group was not added (drug control group, CK). Copper contents in blood and liver in sheep were measured every 20 days during supplementary feeding with ammonium molybdate. And blood physiological and biochemical indexes were measured again at the end of the ammonium molybdate supplementation experiment.

Sample Collection

Soil and Herbage Sample

Twenty 200-g soil samples were collected from the surface layer (0-30 cm) of each pasture, then dried at 60-80ºC for 48 h, and passed through a 0.154-mm sieve. Herbage samples (200 g) were also collected from each pasture. To reduce the influence of soil contamination, the herbage samples were harvested 1-2 cm above ground level, then dried at 60-80ºC for 48 h and passed through a 0.071-mm sieve.

Blood Sample

The jugular vein blood of sheep was collected by aseptic vacuum blood sampling. Each sheep was 20 mL, of which 10 mL was used to determine the blood mineral content, and another 10 mL was used to determine physiological and biochemical indexes. According to Yao et al., the angle and length of the rapid biopsy needle passing through the right hepatic region skin was 45°and 6 cm respectively to obtain liver samples [10].

Determination and Method

Mineral Element Content

Content of manganese (Mn), zinc (Zn), cobalt (Co), copper (Cu), molybdenum (Mo), and selenium (Se) in the pastures were determined by atomic absorption spectrometry (model no. XDY-2A; PerkinElmer, Inc., Waltham, MA, USA) [11]. The ammonium molybdate spectrophotometric method determined element phosphorus (P) [12].

Blood Physiological and Biochemical Indicators

Hemoglobin (Hb), red blood cell count (RBC), hematocrit (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and white blood cell count (WBC) in blood were determined using an animal-specific automatic blood analyzer (SF-3000, Sysmex Corporation, Kobe, Japan).

Blood levels of glutathione peroxidase (GSH-Px), glutamic oxaloacetic transaminase (GOT), catalase (CAT), creatine phosphokinase (CPK), superoxide dismutase (SOD), malondialdehyde (MDA), and ceruloplasmin (CP) were determined using an automatic biochemical analyzer (Olympus AU 640, Olympus Corporation, Tokyo, Japan).

Data Analysis

All data analyses were conducted using IBM SPSS Statistics for Windows software, version 20.0 (IBM Inc., Armonk, NY, USA), and the results were presented as the "mean±standard error". Significant differences between groups were identified using Student's t-test. A probability (*P*) value of <0.01 was considered statistically significant.

Results

Mineral Element Content

The pasture was severely affected, with copper contents in soil significantly exceeding that in the control pasture ($P<0.01$) and the national environmental quality standards of copper pollution in China (35 mg/kg, GB15618-1995). The copper content in herbage of affected pasture was also significantly greater than that in the control pasture ($P<0.01$) (Table 1). There were no significant differences in the content of other elements in soil and herbage between the two pastures. The ratio of copper and molybdenum was significantly greater in herbage of the affected pasture than that in common animal feed (23.05:1 vs. 6-10:1, respectively) [13]. Copper content in blood and liver in the affected group were significantly higher than those in control group ($P<0.01$) (Table 2), with no significant difference in other elements ($P>0.05$). According to Wang et al., when the content of copper in liver exceeds 500 mg, it can be regarded as copper poisoning [14]. Therefore, Wumeng semi-fine wool sheep grazing on affected pasture were in a copper poisoning state.

Biophysical and Biochemical Parameters

Blood levels of Hb, RBC, PCV and MCV in the affected group were significantly lower than those in control ($P<0.01$, Table 3). There was no significant difference in the levels of MCH, MCHC and WBC between the affected and control sheep ($P>0.05$). SOD, CAT and GSH-Px activities in blood in the affected group were significantly lower than control ($P<0.01$) (Table 4), while Cp, GOT, CPK and MDA were significantly greater ($P<0.01$) (Table 4).

Table 1. Mineral contents of soil and herbage mg/kg (DM).

Element	Soil		Herbage	
	Affected pasture	Control pasture	Affected pasture	Control pasture
Mn	312.67±27.56	289.29±25.36	62.79±11.91	57.93±5.67
Zn	45.17±11.67	44.19±9.53	87.68±13.61	87.69±7.83
Co	6.15±1.27	6.13±1.35	1.52±0.63	1.49±0.67
Cu	71.83±7.7 3a	11.87±2.63b	26.97±3.29	7.27±2.73
P	447.77±37.68	438.97±34.45	457.89±33.72	453.29±31.76
Mo	1.76±0.57	1.9681±0.53	1.17±0.13	1.21±0.15
Se	0.123±0.015	0.129±0.016	0.117±0.032	0.123±0.031

Copper = Cu, molybdenum = Mo, manganese = Mn, selenium = Se, cobalt = Co, iron = Fe, zinc = Zn

Note: Different little letters show extremely significant difference ($P<0.01$).

Table 2. Mineral content of blood and liver mg/kg.

Element	Blood		Liver	
	Affected animals	Healthy animals	Affected animals	Healthy animals
Mn	0.57±0.17	0.56±0.19	4.31±0.37	4.29±0.39
Zn	7.83±0.19	7.79±0.89	71.49±13.21	71.39±17.17
Co	0.63±0.17	0.64±0.13	7.25±1.22	7.18±1.23
Cu	8.57±0.37a	0.97±0.11b	802.76±52.77a	94.19±21.17b
P	233.67±21.79	241.83±21.77	612.89±35.71	613.97±32.94
Mo	0.08±0.13	0.09±0.11	3.17±0.79	3.17±0.83
Se	0.147±0.061	0.153±0.032	1.193±0.061	1.174±0.315

Copper = Cu, molybdenum = Mo, manganese = Mn, selenium = Se, cobalt = Co, iron = Fe, zinc = Zn
Note: Different little letters show extremely significant difference (P<0.01).

Effect of Mo in Feed on Cu Content in Blood and Liver

Copper contents in blood and liver were greatly increased, impairing appetite, lassitude and so on symptoms caught up with sheep after the 30 d of transfer stocking in the pasture of affected pasture from the control pasture. Molybdenum supplementation ameliorated these disease symptoms, which eventually disappeared by the end of the trial. The therapeutic effects of different supplementary doses were different (Fig. 1). From Fig. 1 we can see that the copper content in blood and liver had no significant change of Wumeng semi-fine wool sheep in the drug control group (CK group), and the symptoms of dyspnea, jaundice and hemoglobinuria appeared. Among the three groups added with ammonium molybdate, 300 mg/kg had the fastest effect. After 20 days of feeding, the copper content in blood decreased by more than 50%. After 40 days of continuous feeding, the copper content in blood was 1.28 mg/kg and was restored to the normal range of 0.7-1.3 mg/kg. The 100 and 200 mg/kg groups added with molybdenum needed around 40 days to reduce the copper content in blood by 50%, and the time required to reduce the copper content in blood to the normal range was longer. It can be seen that supplementing ammonium molybdate twice every 10 days and 300 mg/kg each time is the best way to solve copper poisoning in Wumeng semi-fine wool sheep in this area.

Effect of Mo in Feed on Blood Physiological and Biochemical Indicators

At the end of the ammonium molybdate supplementation experiment, Hb, RBC and MCH in blood in the ammonium molybdate supplementation groups were significantly higher than those in the CK group (P<0.01), and there was no significant difference among PCV, MCHC and WBC in the four groups (P>0.01) (Table 5). The activity of SOD, CAT, and

Table 3. Physiological parameters in blood.

Blood parameters	Affected animals	Healthy animals
Hb (g/L)	82.67±12.13a	123.37 ± 13.58b
RBC (10^{12}/ L)	6.75±2.87a	11.69 ± 1.35b
PCV (%)	22.57±2.35a	36.87± 5.36b
MCV (fl.)	2.57±0.37a	3.19 ±0. 43a
MCH (pg.)	12.35±2.61	10.65 ± 3.17
MCHC (%)	3.16±0.37	3.33± 0.37
WBC (10^9/ L)	10.77±1.72	9.25 ± 2.37

Hemoglobin = Hb, red blood cell count = RBC, hematocrit = PCV, mean corpuscular volume = MCV, mean corpuscular hemoglobin = MCH, mean corpuscular hemoglobin concentration = MCHC, white blood cell count = WBC
Note: Different little letters show extremely significant difference (P<0.01).

Table 4. Biochemical parameters in blood.

Biochemical parameters	Affected animals	Healthy animals
SOD (IU/mL)	32.87±3.91a	71.67 ± 7.53b
CAT (IU/mL)	0.75±0.27a	1.23 ± 0.31b
GSH-Px (IU/mL)	13.23±6.31a	33.76± 3.73b
Cp (mg/dL)	8.76±1.33a	4.49 ± 1.53b
MDA (nmol/mL)	36.47±3.35a	27.18 ± 5.34b
CPK (IU/mL)	101.76 ± 21.67a	35.77±3.75b
GOT (IU/mL)	47.87 ± 3.39a	16.48±3.87b

Glutathione peroxidase = GSH-Px, glutamic oxaloacetic transaminase = GOT, catalase = CAT, creatine phosphokinase = CPK, superoxide dismutase = SOD, malondialdehyde = MDA, ceruloplasmin = Cp
Note: Different little letters show extremely significant difference (P<0.01).

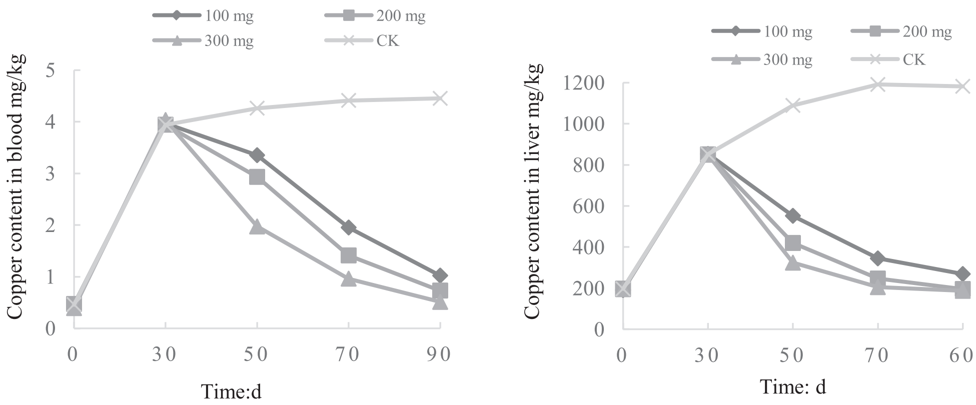

Fig. 1. Effect of ammonium molybdate on copper content in blood and liver.

GSH-Px in blood in ammonium molybdate supplementation groups were significantly higher than those in the CK group (P<0.01), while the activity of CPK, GOT, CP and MDA in blood in ammonium molybdate supplementation groups were significantly lower than those in the CK group (P<0.01) (Table 6).

Discussion

Mineral elements, as inorganic nutrients are a major category of animal nutrients, play an important role in the animals' growth and development, function maintenance, quality of product and immune function and so on [15-17], but when the content exceeds the requirement, it will show certain toxicity [18, 19]. The soil has to provide the mineral nutrients for the plants, and the plants in turn must make the nutrient elements absorbed from the soil available to the animal. The evidence of interdependence among the soil, plant and animal should be the basis for the provision of an adequate source of food for man [20]. Therefore, the accumulation of heavy metals in the environment not only directly influence animal health, but also affect human health through the food chain [21, 22]. In this study, copper content in blood and liver in the sheep that grazed on the copper-polluted meadow was significantly higher than that in the control pasture. This indicated that excessive copper in the environment had an effect on copper in the sheep.

The red blood cell count and hemoglobin content decreasing have the characteristics of anemia [23]. The red blood cell count and hemoglobin content of sheep grazing on copper-contaminated grassland were significantly lower than those in control group (P < 0.01), which was consistent with anemia observed in Wumeng sheep.

Aerobic organisms often produce a large number of reactive oxygen species such as superoxide anion radical (O_2^-), hydroxyl radical (-OH), and hydrogen peroxide (H_2O_2) in the oxidation reduction cycle. If too many free radicals cannot be removed in time, they will attack various biological macromolecules, causing a series of oxidative damage such as DNA damage, enzyme inactivation, lipid peroxidation and so on, and then causing various physiological pathological changes of organisms [24-26]. In the long-term evolution process, organisms have formed a complete protective antioxidant system to remove excess free radicals in the body [27]. The system includes non-enzymatic antioxidants and enzymatic antioxidants. Non-enzymatic antioxidants are mainly vitamins E, vitamin C, glutathione, nitric oxide, β-carotene, etc. Enzyme antioxidants include superoxide dismutase, catalase and glutathione peroxidase [28, 29]. Superoxide dismutase plays the first role in scavenging reactive

Table 5. Physiological indexes in blood after adding ammonium molybdate.

Levels of ammonium molybdate (mg/kg)	Hb (g/L)	RBC (10^{12}/ L)	PCV (%)	MCV (fl.)	MCH (pg.)	MCHC (%)	WBC (10^9/L)
0	80.41±6.17a	6.20±1.14a	23.23±2.09a	2.53±0.59	12.19±1.56	3.03±0.59	10.77±1.73
100	106.06±15.44 b	10.54±1.27b	34.31±1.69 b	2.81±0.52	10.77±1.21	2.91±0.52	10.80±0.92
200	118.23±15.31 b	10.93±0.85b	34.51±1.88 b	3.01±0.43	11.57±1.21	3.07±0.43	10.24±1.98
300	119.67±10.04 b	11.67±1.48b	35.46±1.67b	3.15±0.49	10.62± 3.19	3.15±0.49	9.64±2.54

Hemoglobin = Hb, red blood cell count = RBC, hematocrit = PCV, mean corpuscular volume = MCV,
mean corpuscular hemoglobin = MCH, mean corpuscular hemoglobin concentration = MCHC, white blood cell count = WBC
Note: Different little letters show significant difference (P<0.01).

Table 6. Biochemical indexes in blood after adding ammonium molybdate.

Levels of ammonium molybdate (mg/kg)	SOD (IU/mL)	CAT (IU/mL)	GSH-Px (IU/mL)	CP (mg/dL)	MDA (nmol/mL)	CPK (IU/mL)	GOT (IU/mL)
0	31.05±2.86a	0.65±0.21a	12.63±3.06a	8.64±1.32a	33.38±3.22a	102.40±8.32a	47.75±4.26a
100	62.78±6.87b	1.04±0.15b	30.37±2.26b	5.01±0.94b	27.31±2.08b	36.22±4.44b	19.34±2.06b
200	67.48±4.00b	1.15±0.27b	32.95±3.66b	4.81±1.09b	28.28±3.28b	33.98±5.87b	19.14±2.86b
300	71.21±4.20b	1.23±0.18b	33.53±3.69b	4.93±1.06b	27.22±2.91b	34.81±7.19b	17.74±1.36b

glutathione peroxidase = GSH-Px, glutamic oxaloacetic transaminase = GOT, catalase = CAT, creatine phosphokinase = CPK, superoxide dismutase = SOD, malondialdehyde = MDA, ceruloplasmin = Cp
Note: Different little letters show significant difference (P<0.01).

oxygen species (ROS). It rapidly disproportionates superoxide anion radicals into hydrogen peroxide and molecular oxygen. Hydrogen peroxide is converted into water and molecular oxygen under the action of catalase and glutathione peroxidase. Therefore, superoxide dismutase, catalase and glutathione peroxidase can scavenge oxygen-free radicals and protect cells from oxidative damage. The studies on the effects of high copper and copper poisoning on animal blood indicators have found that high copper can increase superoxide dismutase, ceruloplasmin and glutathione peroxidase activities in liver and blood, but can inhibit superoxide dismutase and glutathione peroxidase activity after copper poisoning [30, 31]. There were significant differences in the blood parameters in copper-poisoning sheep illustrated by elevated levels of serum aspartate aminotransferase (AST), creatine phosphokinase, and gamma-glutamyltransferase (GGT) activities [32]. In this experiment, the blood glutathione peroxidase activity of sheep in the affected group was lower than that in the control group, which indicated that a copper-contaminated environment has an effect on the antioxidant system of sheep. Besides, the activities of malondialdehyde and glutamic oxaloacetic transaminase in blood in the affected group were significantly higher than those in the control group, which was the result of copper poisoning promoting the production of lipid peroxides and liver injury.

After adding ammonium molybdate, copper in blood and liver decreased, hemoglobin and red blood cell count content and glutathione peroxidase activity increased, and malondialdehyde and glutamic oxaloacetic transaminase activity decreased. This is consistent with the result of previous studies that molybdenum blocks copper absorption, highlighting the antagonism between molybdenum and copper, and forms thiomolybdate in the gastrointestinal tract [33-35], in addition to the formation of Cu-thiomolybdate complexes and Cu-Mo-S-protein compounds [36, 37]. Cu-thiomolybdate complexes are insoluble and excreted directly from the intestinal tract, which blocks the absorption of copper, resulting in decreased copper levels. Cu-Mo-S-protein compounds are more stable in the blood and copper is not easily utilized by tissue. In the liver, the thiomolybdate anions can directly peel copper away from the metallothionein protein, and the peeled copper enters the blood and bile by way of other small metalloproteins that then transfer the copper to other proteins, and so on, which results in the gradual decrease of copper stores in the liver of sheep under grazing conditions and eventual depletion. In the "digestive tract effect-systematical effect" proposed by Mason Smith when describing the interactions among three species, it was also mentioned that molybdate in feed can react with sulfur compounds to produce thiomolybdate in the rumen of ruminants, which then reacts with copper to produce insoluble Cu-thiomolybdate complexes so as to reduce the absorption of copper. Secondly, protein-Cu-Mo compounds are formed, but cannot be utilized physiologically by combining some form of absorbed molybdenum with plasma proteins and copper in the plasma so as to reduce the absorption of copper. Therefore, molybdenum supplementation can reduce copper absorption of Wumeng semi-fine wool sheep and prevent chronic copper poisoning caused by excessive long-term copper intake. However, excessive molybdenum supplementation will cause an imbalance in the Mo:Cu ratio, which will also have a serious impact on animal health [38-40]. Moreover, excessive molybdenum supplementation of the diet is a known cause of secondary copper deficiency-related diseases and an inappropriate proportion of molybdenum-to-copper in ruminant feed may also cause symptoms of molybdenum poisoning, such as anorexia, growth stagnation, weight loss, anemia, depilation, dermatitis, loss of hair color, and spontaneous fracture. The suitable Mo:Cu ratio in feed is 6-10:1. An Mo:Cu ratio of less than 2:1 will cause an increase in molybdenum absorption and enhance the reabsorption of metabolic molybdenum by the renal tubules [41, 42]. Therefore, in order to prevent the adverse effects caused by the maladjustment of the Mo:Cu ratio due to excessive supplementation of molybdenum in feed, it is necessary to monitor copper intake and to supplement molybdenum according to the appropriate proportion of copper and molybdenum.

Conclusions

Copper-contaminated grassland increased the content of copper in blood and liver of grazing Wumeng semi-fine wool sheep, which had a negative impact on blood physiological and biochemical indicators. Supplementation of ammonium molybdate in diet (2 every 10 days, 300 mg/kg each time) can restore abnormal blood parameters to normal. Therefore, utilize the antagonism of molybdenum and copper in the diet to achieve the goal of harmless utilization of the copper-polluted meadow. We should pay attention to the rational formulation of feed for the non-infected sheep to avoid diseases caused by excessive nutrients or lack of them.

Acknowledgments

This work was supported as a project of the National Key Research and Development Program of China in the 13th Five-Year Plan (2016YFC0502601), the National Natural Science Foundation of China (41671041), and the Doctoral Program Foundation of Southwest University of Science and Technology of China (17zx7146).

Competing Financial Interests

The authors declare they have no present or potential competing financial interests.

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