Studies of High Molybdenum-Induced Copper Deprivation in P. przewalskii on the Qinghai Lake Pasture in China

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Abstract: The Przewalski’s Gazelles (P. przewalskii) are affected by disorders that are characterized by deprived appetites, pica, emaciation and dyskinesia. The purpose of this study was to investigate the possibility of high molybdenum (Mo) in forage leading to copper (Cu) deprivation. The mineral contents in forage, soil, and samples of hair and blood from affected ranges were compared to healthy pasture. Blood parameters were also determined. Our results showed that the mean content of Mo in forage and soil in studied pasture was 5.17 and 4.17 µg/g, respectively. The Cu to Mo ratio in forage from affected and healthy areas was 1.26 and 5.89 µg/g, respectively. The Cu concentrations in hair and blood from gazelles in affected pasture were extremely lower (p < 0.01) than those in unaffected animals. The Mo contents in hair were higher (p < 0.01) than those in unaffected gazelles. The levels of Hb, RBC, PCV, MCV, and MCH in unaffected gazelles were significantly lower (p < 0.01) than those in unaffected gazelles. The levels of TP, ALB and GLB in blood were significantly lower (p < 0.01) than those in unaffected gazelles, while the levels of AST, LDH, CPK and ALP in serum were significantly higher (p < 0.01) than those in unaffected gazelles. The activities in serum T-AOC, SOD, GSH-Px, and CAT in affected gazelles were extremely lower (p < 0.01) than those in unaffected gazelles, while MDA was significantly higher (p < 0.01) than that in unaffected gazelles. Supplementation in copper sulphate (CuSO4) has prevented and cured this disorder. In summary, high molybdenum in feed and soil may lead to secondary Cu deficiency in gazelles, which can be alleviated by supplement of copper sulfate.

Keywords: pasture management; copper deprivation; P. przewalskii; molybdenum; the Qinghai Lake watershed

1. Introduction

The Przewalski’s Gazelles (P. przewalskii) are endemic to the Qinghai–Tibet Plateau in China, and are widely distributed throughout the Xizang, Inner Mongolia, Gansu, Ningxia, Qinghai, Shaanxi, and Xinjiang [1,2]. The Qinghai Lake Watershed has become the last natural habitat of gazelles, because of environmental change, human population growth and habitat fragmentation [3–5]. At present, wild populations are mainly distributed in several isolated points (Bird Island sanctuary, Hudong-Ketu pasture, and Yuanzhe pasture) in the Qinghai Lake Pasture [6,7]. Among endemic mammals, wild gazelles have been the species with the least population [8]. The endangered status prompted the International Union for Conservation in Nature (IUCN) to list gazelles as an endangered species [9].

Copper (Cu) is an essential element for animals [10,11]. Cu is deficient in many parts of the world, and there are many pastures that are not suitable for grazing ruminants [12]. In Britain, the analysis of serum Cu content in cattle showed that a series of diseases were related to Cu deficiency [13]. The annual incidence rate of Cu deprivation is about
0.9% of the total. In the incidence area, although the mortality rate was low, the main economic loss was that the animal could not grow healthfully [14–16], and the incidence of lamb ataxia in some areas was 30% [17]. The Cu deprivation included induced or direct types in animals [18]. Induced deprivation occurs when Cu, molybdenum (Mo), and sulphur (S) combine in rumen to form Cu-thiomolybdate complexes that are very poorly absorbed. The high S intakes can also greatly decrease Cu status, independent of Mo, and reduce feed intake [19,20]. Direct deprivation occurs when Cu contents in forage are significantly lower than the healthy level. The clinical symptoms in Mo-induced Cu deprivation in animals, including anorexia, diarrhea, poor growth, pica, emaciation, and dyskinesia [21–23] and swayback were seen in gazelles in the Qinghai Lake watershed, which were known colloquially as “swayback ailments”.

Our aim in this experiment is to investigate the possibility of high molybdenum in soil and feed may lead to secondary Cu deficiency, and the effects of Cu nutrition supplementation on gazelles, and to provide reference for further study of the Cu-deficient ailment in gazelles.

2. Materials and Methods

2.1. Studied Pasture

The studied pastures are located in the eastern part of the Qinghai Lake Pasture in Qinghai-Tibetan Plateau of China (35°43′–36°33′ N, 102°46′–103°29′ E), where the average elevation is 3200 m above sea level. The difference in temperature is very large, and the frost-free period is only 95 days. Steppe and alpine meadows are the major vegetation. The main plant types are Stellera chamaejasme, Agropyron cristatum, Puccinellia distans, Achnatherum splendens, Artemisia desertorum, Iris lacteal, and Orinus kokonorica [24].

2.2. Experimental Design

Twenty gazelles (1.5 year) were selected from the Bird Island Area, where the diseases had still not been reported. The gazelles were judged to be in good health in examinations and were then used as control animals. Twenty gazelles (1.5 year) from affected grassland in Hudong pasture, where animals showed signs of “swayback ailment”, were also selected as treatment animals. Ten gazelles from the affected pasture were treated orally with copper sulfate (CuSO₄), 6 g/animal once every 10 days. The other 10 affected animals were grazing on affected grassland without any treatment. Test duration was 20 days.

2.3. Collection Samples

Ten soil samples in the studied pasture were collected from the surface layer (0 to 30 cm), marking sampling points. Ten samples of mixed herbage were also collected from each studied area. In order to reduce soil pollution, the samples of forages were cut 1–2 cm above ground level.

Samples of hair from selected animals were also taken on the gazelles’ necks. Samples were washed with acetone and rinsed six times with deionized water, and samples were kept on a silica gel in the desiccator until analysis. The whole blood samples were obtained from the jugular vein into vacuum blood-collection tubes with EDTA-K₂ anticoagulant. Anticoagulant tubes were kept at 4 °C until assay of hematology examination. Blood samples were collected using vacuum blood collection tubes with 1% sodium heparin as anticoagulant, then stored at −20 °C for analysis of trace elements. Serum samples collected using a separation gel accelerating tube for biochemical analysis were centrifuged at 3500 × g for 10 min, then stored in an EP tube at −80 °C until analyzed.

2.4. Analysis and Treatment of Samples

Mineral concentrations in samples of forage, soil, hair, and blood are determined using inductively coupled plasma atomic emission spectrometry (ICP-AES). The samples of soil, forage and animal tissues were digested by microwave; 0.1–0.2 g samples were accurately weighed in a microwave digestion tank, moistened with a small amount of
deionized water, and then 6 mL HNO\textsubscript{3} and 2 mL HF was added. After cooling, the solutions were transferred to 50 mL PTFE crucibles, and 3 mL HClO\textsubscript{4} was added. The temperature of the electric heating plate was controlled at 180 °C. When heated to thick white smoke, the solutions were covered until the organic matter on the wall of the crucible disappeared. The cover was opened to drive away the white smoke and steam until the digestion solution was viscous. The crucible was removed and cooled slightly. Then the soluble residues were added, with 2 mL HCl to be dissolved. The inner wall was washed with deionized water and then the whole amount was transferred to a 50 mL volumetric flask for determination. The microwave digestion conditions were acid system (7 mL HNO\textsubscript{3} and 2 mL HF), digestion temperature (180 °C), and holding time (20 min). The best working conditions of the ICP-AES instrument were RF power (1100 W), cooling gas flow (0.95 L·min\textsuperscript{-1}), carrier gas flow (0.95 L·min\textsuperscript{-1}), peristaltic and pump speed (20 rpm).

Analyzed mineral elements were Fe, Mo, Cu, Zn, manganese (Mn), and cobalt (Co). Hemoglobin (Hb), erythrocyte count (RBC), white cell count (WBC), packed cell volume (PCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin content (MCHC), and mean corpuscular volume (MCV) were determined using an automatic blood cell analyzer (SF-3000, Sysmex-Toa Medical Electronics, Kobe, Japan). Biochemical values, including blood urea nitrogen (BUN), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), alanine transaminase (ALT), creatine phosphate kinase (CPK), alkaline phosphatase (ALP), total protein (TP), creatinine (CR), globulin (GLB), cholesterol (Chol), albumin (ALB), glutathione peroxide (GSH-Px), albumin/globulin (A/G), superoxide dismutase (SOD), catalase (CAT), total antioxidant capacity (T-AOC), and malondialdehyde (MDA) contents were determined by an automatic biochemical analyzer (SF1, Shanghai City Medical Apparatus and Instruments Factory, Shanghai, China).

2.5. Statistical Analyses

All data in our experiment were analyzed by a statistical package (SPSS, version 21.0, Inc., Chicago, IL, USA). All data will be presented below as means ± SD, and data values are considered to be statistically significant at \( p < 0.01 \).

3. The Results

3.1. Mineral Concentrations

As shown in Table 1, the concentrations of Mo in forage and soil in the affected pasture were markedly higher (\( p < 0.01 \)) than those in the healthy area. The Cu to Mo ratio in soil in the affected and healthy areas were 3.51:1 and 12.79:1, respectively, and those in forage in the affected and healthy areas were 1.28:1 and 5.89:1, respectively. Other element contents were within the healthy ranges.

| Elements | Soils (µg/g) | Forages (DM) (µg/g) |
|----------|-------------|---------------------|
|          | Affected    | Unaffected          | Affected    | Unaffected          |
| Cu       | 14.63 ± 2.41 | 14.75 ± 2.42        | 6.53 ± 0.59 | 6.71 ± 0.66         |
| Mo       | 4.17 ± 0.20 * | 1.15 ± 0.23        | 5.17 ± 0.15 * | 1.14 ± 0.13        |
| Fe       | 4247.35 ± 25.97 | 4255.67 ± 23.72 | 335.25 ± 5.36 | 332.65 ± 6.86 |
| Mn       | 58.97 ± 6.28 | 57.17 ± 5.97        | 12.97 ± 1.25 | 12.65 ± 1.76       |
| Co       | 5.76 ± 0.86  | 5.68 ± 0.68         | 1.15 ± 0.06  | 1.14 ± 0.07        |

* Indicates great difference at \( p < 0.01 \). DM = dry matter.

The contents of mineral nutrition from samples of blood and hair are given in Table 2. Cu concentrations in samples of blood and hair in gazelles from the affected pasture were extremely lower (\( p < 0.01 \)) than those in healthy gazelles. The Mo concentrations from samples of hair in affected gazelles were significantly higher (\( p < 0.01 \)) than those in healthy gazelles. Other element contents were within the healthy ranges.
Table 2. The mineral element in blood and hair in gazelles (µg/g).

| Elements | Blood | Hair |
|----------|-------|------|
|         | Affected | Unaffected | Affected | Unaffected |
| Cu      | 0.18 ± 0.03 * | 1.61 ± 0.63 | 3.99 ± 0.33 * | 7.29 ± 0.37 |
| Mo      | 0.39 ± 0.03 * | 0.23 ± 0.02 | 4.35 ± 0.06 * | 2.49 ± 0.04 |
| Fe      | 442.67 ± 62.38 | 439.65 ± 63.47 | 318.65 ± 43.26 | 316.75 ± 45.64 |
| Mn      | 0.53 ± 0.02 | 0.52 ± 0.03 | 11.65 ± 1.58 | 11.87 ± 1.86 |
| Co      | 0.55 ± 0.03 | 0.54 ± 0.05 | 1.03 ± 0.04 | 1.02 ± 0.03 |

* Indicates great difference at \( p < 0.01 \).

3.2. Physiological Parameter and Biochemical Indexes in Blood

The values of Hb, RBC, MCH, PCV, and MCV in gazelles from the affected pasture were significantly lower \( (p < 0.01) \) than those in unaffected gazelles (Table 3).

Table 3. Blood hematological levels in gazelles.

| Parameters | Affected Gazelles | Healthy Gazelles |
|------------|-------------------|------------------|
| Hb (g/L)   | 90.76 ± 7.41 *    | 119.63 ± 8.87    |
| RBC (10^{12}/L) | 5.73 ± 0.33 *       | 7.99 ± 0.25       |
| PCV (%)    | 39.63 ± 3.76 *    | 53.63 ± 3.58      |
| MCV (fL)  | 47.35 ± 3.21 *    | 53.47 ± 3.76      |
| MCH (pg)   | 10.74 ± 1.64 *    | 16.38 ± 1.56      |
| MCHC (%)  | 22.76 ± 1.37      | 24.31 ± 1.57      |
| WBC (10^{9}/L) | 8.48 ± 0.58       | 9.76 ± 0.52       |

* Indicates great difference at \( p < 0.01 \).

The levels of TP, ALB and GLB in blood were significantly lower \( (p < 0.01) \) than those in unaffected gazelles, while levels of AST, LDH, CPK, and ALP in animal blood were extremely higher \( (p < 0.01) \) than those in unaffected gazelles (Table 4).

Table 4. Physiological and biochemical levels in blood in gazelles.

| Items       | Affected Gazelles | Healthy Gazelles |
|-------------|-------------------|------------------|
| AST (U/L)   | 132.47 ± 11.22 * | 89.98 ± 8.37     |
| ALT (U/L)   | 45.77 ± 4.35      | 44.77 ± 3.57     |
| LDH (U/L)   | 553.48 ± 34.58 * | 425.73 ± 30.75   |
| CPK (U/L)   | 386.37 ± 63.86 * | 255.37 ± 54.97   |
| ALP (U/L)   | 1223.76 ± 207.83 * | 751.28 ± 93.56 |
| CR (µmol/L) | 65.56 ± 6.67      | 66.74 ± 5.27     |
| TP (g/L)    | 38.63 ± 4.48 *    | 56.38 ± 3.86     |
| ALB (g/L)   | 24.86 ± 3.58 *    | 32.86 ± 2.36     |
| GLB (g/L)   | 13.86 ± 2.49 *    | 24.86 ± 2.88     |
| A/G         | 1.84 ± 0.21 *     | 1.29 ± 0.14      |
| Chol (mmol/L) | 1.97 ± 0.21       | 1.89 ± 0.23      |

* Indicates great difference at \( p < 0.01 \).
Table 5. The antioxidant levels in serum in gazelles.

| Items        | Affected Gazelles | Healthy Gazelles |
|--------------|-------------------|------------------|
| T-AOC (U/mL) | 2.23 ± 0.11 *     | 4.75 ± 0.21      |
| GSH-Px (U/mL)| 227.54 ± 11.77 *  | 351.65 ± 17.36   |
| MDA (nmol/L) | 42.48 ± 4.27 *    | 13.76 ± 2.26     |
| SOD (U/mL)   | 58.37 ± 4.52 *    | 99.72 ± 9.96     |
| CAT (U/mL)   | 6.76 ± 0.21 *     | 14.36 ± 1.76     |

* Indicates great difference at $p < 0.01$.

3.3. Mineral Levels in Animal Blood

As shown in Table 6, the level of Cu in animal blood was significantly increased in treatment gazelles ($p < 0.01$) and reached a healthy level at the 10th day. Cu concentrations in blood had no significant change in control gazelles. No significant changes in other element contents were observed in treatment gazelles.

Table 6. The mineral element level in animal blood ($\mu$g/g).

| Elements | Treatment Group | Control Group |
|----------|----------------|---------------|
|          | 0 d  | 10 d  | 20 d  | 0 d  | 10 d  | 20 d  |
| Cu       | 0.17 ± 0.04 * | 1.53 ± 0.17 * | 1.77 ± 0.16 * | 0.17 ± 0.03 | 0.16 ± 0.03 | 0.14 ± 0.04 |
| Mo       | 0.22 ± 0.02  | 0.19 ± 0.02  | 0.17 ± 0.01  | 0.24 ± 0.03 | 0.22 ± 0.04 | 0.21 ± 0.03 |
| Fe       | 441.43 ± 61.87 | 443.75 ± 62.38 | 442.86 ± 59.32 | 441.65 ± 60.65 | 439.68 ± 63.48 | 439.65 ± 67.86 |
| Mn       | 0.53 ± 0.02  | 0.52 ± 0.01  | 0.52 ± 0.02  | 0.54 ± 0.03 | 0.53 ± 0.02 | 0.51 ± 0.02 |
| Co       | 0.54 ± 0.04  | 0.54 ± 0.04  | 0.56 ± 0.02  | 0.55 ± 0.04 | 0.57 ± 0.03 | 0.56 ± 0.03 |

* Indicates great difference at $p < 0.01$.

4. Discussion

Many studies have shown that Mo levels less than 3 or 2 $\mu$g/g (DM) in forage and soil samples, respectively, are very safe for ruminants [25]. In our study, Mo levels were much higher ($p < 0.01$) than those in the healthy pasture. At the same time, an increase of Mo content in forage will affect the absorption of Cu by animals. The Cu contents in hair and blood in affected gazelles were lower ($p < 0.01$) than those in unaffected gazelles, while Mo levels were higher ($p < 0.01$) than those in healthy gazelles. This leads to secondary Cu deprivation caused by body absorption disorder [26–28]. The Cu contents in affected gazelles were 0.17 $\mu$g/g, but the average blood Cu values of <0.50 $\mu$g/g are a sign of severe Cu deficiency for ruminants [6]. The Cu concentration in hair is also a common index to evaluate mineral nutrition status of animals [29,30]. The mean level of Cu in hair sample in affected gazelles of 3.99 ± 0.33 $\mu$g/g was also well below the 5.5 $\mu$g/g level of secondary Cu deprivation [6,9]. In addition, the Cu concentrations in hair and blood of affected gazelles reached the normal level after oral administration of CuSO$_4$ until the 10th day. Thus, it was reasonable to conclude that the ailment in gazelles in the Qinghai Lake watershed might be Cu deprivation caused by the high Mo content in forage.

Many studies in animals and humans have shown that there is a significant correlation between Cu deprivation and anemia [31,32]. In this study, the blood routine parameters showed that the values of RBC, Hb and PCV in Cu-deprived gazelles were very decreased, indicating that Cu deprivation had different effects on blood cells of gazelles. The levels of AST, ALT and ALP in blood were commonly used as indices to evaluate animal liver health, and the activities of LDH and CPK in blood were commonly used as indices to reflect the degree of muscle and kidney injuries. The content of ALB changed with the change of feed protein. The content of ALB decreasing indicated that there was a lack of protein, and it might also be the decrease of ALB synthesis caused by liver diseases. GLB was synthesized by the immune organs of animals, most of which were produced outside the liver cells, but also related to the immune system of the organism [33,34]. The concentrations of ALB and GLB complement each other to maintain a constant level of total protein [35]. The activities
of AST and ALP in the blood of Cu-deprived gazelles were increased, which suggested that the hepatocytes of animals were damaged and the permeability was increased. The increased activities of CPK and LDH indicated that kidney and muscle cells of gazelles were damaged. The levels of TP, ALB and GLB in blood decreasing significantly indicated that the immune system of gazelles was affected. The main reason for the damage to liver, kidney and muscle caused by Cu deficiency was related to the weakening of the antioxidant system [36–38].

The antioxidant protection system in animals is a defense system to scavenge free radicals, including enzymatic and non-enzymatic systems [39,40]. The non-enzymatic system includes mainly vitamins, glutathione (GSH), Mn, iron (Fe), and selenium (Se). The enzymatic system includes mainly CAT, GSH-Px, SOD, and other antioxidant enzymes [41]. The antioxidation effect in animals is to remove excess free radicals and maintain the body balance between oxidation and anti-oxidation. SOD can effectively convert O$_2^-$ to H$_2$O$_2$, which is cleared by GSH-Px and CAT [42]. The function of GSH-Px is to reduce lipid hydrogen peroxide to corresponding alcohol and free H$_2$O to water [43]. MDA is the product of lipid peroxidation, and its level in the body can directly reflect the degree of lipid oxidative damage [44]. T-AOC is a comprehensive index to determine the antioxidant function of an organism, which can reflect the organism compensatory ability to external stimuli and the metabolic ability of free radicals. The decline of the T-AOC defense system can cause a series of diseases [45–47]. Consequently, this study showed that the levels of T-AOC, SOD, GSH-Px, and CAT were significantly decreased and MDA concentration in blood was significantly increased in Cu-deprived gazelles, which fully indicated that Cu deficiency induced the dysfunction of the antioxidant system in gazelles, broke the balance between the oxidative system and the antioxidant system, and led to severe oxidative stress.

5. Conclusions

The results showed that Cu contents in soils and forage are safe for ruminants, but the Mo levels in soil and forage are much higher than those of the unaffected pasture. As a result, Cu contents of blood and hair in affected animals are much lower than those of gazelles in the unaffected pasture. Therefore, the nutrition deprivation in gazelles was a secondary Cu deficiency caused by the high Mo content in the soil and forage.

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