Effects of Dietary Copper Level on Production Performance, Nutrient Apparent Digestibility, and Serum Biochemical Indices in Dairy Cows (Postprint)

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Abstract

This experiment aimed to investigate the effects of dietary copper levels on production performance, nutrient apparent digestibility, and serum biochemical indices in dairy cows. Sixty Chinese Holstein dairy cows with similar milk yield, days in milk, and parity were selected and divided into 4 groups according to the principle of random allocation. The copper supplementation amounts in the diets of each group were 0 (control, Group), 10 (Group), 15 (Group), and 20 mg/kg (Group), respectively, with dietary copper levels of 8.09, 18.09, 23.09, and 28.09 mg/kg DM. Each group had 15 cows, and the experimental period lasted 93 days. The results showed: 1) Compared with Group , the milk yield of Groups , , and increased by 4.91%, 6.27%, and 4.78%, respectively (P<0.05), and the somatic cell count in milk decreased extremely significantly (P<0.01). 2) Compared with Group , the apparent digestibility of neutral detergent fiber (NDF) in Groups and increased by 6.21% and 7.60%, respectively (P<0.05), and the apparent digestibility of acid detergent fiber (ADF) increased by 6.78% and 8.22%, respectively (P<0.05). 3) Compared with Group , the contents of serum copper and ceruloplasmin and the activities of superoxide dismutase (SOD), copper-zinc superoxide dismutase (Cu-Zn SOD), and glutathione peroxidase (GSH-Px) in Groups , , and increased extremely significantly (P<0.01), the malondialdehyde (MDA) content decreased by 50.00%, 60.23%, and 47.08%, respectively (P<0.01), and the immunoglobulin G (IgG) content increased by 49.56%, 165.56%, and 90.22%, respectively (P<0.01). 4) Analysis of copper metabolism indices indicated that, compared with Group , dietary copper supplementation could extremely significantly increase the contents of fecal copper and urinary copper (i.e., the excretion of copper in feces and urine) and copper deposition (P<0.01), but dietary copper level had no significant effect on the ap-

parent digestibility of copper (P>0.05). Under the conditions of this experiment, dietary copper supplementation could improve the production performance of dairy cows, promote the utilization of nutrients by the body, and enhance the immune and antioxidant capacities of the body, but fecal copper increased with increasing dietary copper levels. Taking all factors into consideration, under the conditions of this experiment, the appropriate dietary copper level for dairy cows was 18.09–23.09 mg/kg DM (copper intake was 403.59–514.21 mg/d).

Full Text

Effects of Dietary Copper Levels on Performance, Nutrient Apparent Digestibility, and Serum Biochemical Indices of Lactating Dairy Cows

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Abstract: This study investigated the effects of dietary copper levels on the performance, nutrient apparent digestibility, and serum biochemical indices of lactating dairy cows. Sixty China Holstein cows with similar milk yield, lactation stage, and parity were randomly allocated to four groups (n=15 each). The basal diet was supplemented with copper at 0 (control, Group I), 10 (Group II), 15 (Group III), and 20 mg/kg (Group IV), resulting in dietary copper concentrations of 8.09, 18.09, 23.09, and 28.09 mg/kg DM, respectively. The 93-day trial period included 90 days of feeding and 3 days of digestion-metabolism studies. The results showed: (1) Compared with Group I, milk yield increased by 4.91%, 6.27%, and 4.78% in Groups II, III, and IV, respectively (P<0.05), while somatic cell count in milk decreased significantly (P<0.01). (2) Neutral detergent fiber (NDF) apparent digestibility increased by 6.21% and 7.60% in Groups II and III, respectively (P<0.05), and acid detergent fiber (ADF) apparent digestibility increased by 6.78% and 8.22% (P<0.05). (3) Serum copper and ceruloplasmin concentrations, and superoxide dismutase (SOD), copper-zinc superoxide dismutase (Cu-Zn SOD), and glutathione peroxidase (GSH-Px) activities increased significantly (P<0.01), whereas malondialdehyde (MDA) decreased by 50.00%, 60.23%, and 47.08% (P<0.01) in Groups II, III, and IV, respectively. Immunoglobulin G (IgG) increased by 49.56%, 165.56%, and 90.22% (P<0.01), respectively. (4) Copper supplementation significantly increased fecal and urinary copper excretion and copper deposition (P<0.01), but did not affect copper apparent digestibility (P>0.05). In conclusion, dietary copper supplementation improved lactating performance, nutrient utilization, antioxidant capacity, and immunity, but increased copper excretion. Under the conditions of this study,

the optimal dietary copper level for dairy cows is 18.09–23.09 mg/kg DM (copper intake: 403.59–514.21 mg/d).

Keywords: dairy cows; copper; performance; apparent digestibility; serum biochemical indices; copper metabolism

Introduction

Trace element copper plays a crucial role in lactation and health of dairy cows. With continuous improvements in production levels, producers often add excessive copper to diets to prevent reproductive and performance declines and immune dysfunction associated with copper deficiency [1-2]. Since the early 20th century, copper has been recognized as an essential trace element that participates in enzyme synthesis and serves as an important component and specific activator of various enzymes [3-4]. Yu et al. [5] reported that dietary supplementation with 10 mg/kg copper improved milk fat percentage and milk yield in dairy cows. Liu [6] found that milk yield increased by 20.79% when 16 mg/kg copper was added, but decreased at 24 mg/kg supplementation. Appropriate copper supplementation can increase serum ceruloplasmin concentration and superoxide dismutase (SOD) activity [6-7], though these parameters decline at 24–27 mg/kg copper supplementation [6-8]. Underwood et al. [9] reported that dietary sulfur and molybdenum reduce copper utilization. The copper requirement for a 650-kg cow producing 40 kg of milk has increased from 10 mg/kg (NRC, 1988) [10] to 15.7 mg/kg (NRC, 2001) [11], with some reports suggesting a further 20–50% increase above NRC (2001) levels [12]. Previous studies on copper requirements and effects of different supplementation levels have yielded inconsistent results, with most focusing on serum ceruloplasmin and SOD activity while reporting few other blood parameters, thus failing to comprehensively reveal copper' s effects on physiological status. Additionally, breed improvement and increased production levels may affect copper requirements. This study aimed to investigate the effects of dietary copper levels on performance, nutrient apparent digestibility, and serum biochemical indices to recommend optimal dietary copper concentrations for dairy cows.

Materials and Methods

1.1 Experimental Design and Animals The trial was conducted at Baoding Haoyu Dairy Farm. Sixty healthy Holstein cows in mid-lactation were randomly assigned to four groups (n=15 each) using a single-factor design. Groups had similar parity, milk yield, and lactation days ($P>0.05$). Feed-grade copper sulfate pentahydrate was used as the copper source, with supplementation levels of 0 (control, Group I), 10 (Group II), 15 (Group III), and 20 mg/kg (Group IV), resulting in dietary copper concentrations of 8.09, 18.09, 23.09, and 28.09 mg/kg DM, respectively. The 93-day trial included 90 days of feeding and 3 days of digestion-metabolism studies. The basal diet composition and nutrient

levels are shown in .

1.2 Dietary Management Cows were housed in free-stall barns with *ad libitum* access to feed and water. Feed was delivered twice daily. For each treatment group, premix containing different copper levels was mixed with concentrate before being blended with forage and fed as TMR. Daily milk yield was recorded throughout the trial. Premixes were custom-formulated by Beijing Fuweikang Biotechnology Co., Ltd.

1.3 Sample Collection 1.3.1 Feed Samples
During the trial, feed offered and refused was weighed for three consecutive days each week. Feed samples were collected using the quartering method, air-dried at 65°C for 48 h to constant weight, and stored in sealed plastic bags.

1.3.2 Milk Yield and Samples
Daily milk yield was recorded throughout the trial. Milk samples were collected once monthly, with 60 mL taken during morning, midday, and evening milkings and pooled in a 4:3:3 ratio for component analysis.

1.3.3 Blood Samples
On the day before the trial ended, blood was collected via jugular venipuncture before morning feeding from three cows per group. After 0.5 h in a 37°C water bath, serum was separated by centrifugation (1,240×g, 15 min), aliquoted into 0.5-mL tubes, and stored at -20°C for analysis.

1.3.4 Fecal and Urine Samples
On the final day of the feeding trial, three cows per group were selected for a 3-day digestion-metabolism study using total collection methods. Daily feces were weighed, and 5% of each cow' s daily fecal output was divided into two portions: one preserved with 10% sulfuric acid (20 mL per 100 g feces) and one without acid. Urine was collected in buckets containing 200 mL of 10% sulfuric acid to prevent decomposition, with 1% of total volume sampled and pooled daily. All samples were stored at -20°C until analysis.

1.4 Measurements 1.4.1 Dry Matter Intake (DMI)
Feed offered and refused was recorded for three consecutive days each week. DMI was calculated from feed offered and refused, measured 39 times throughout the trial. Average daily DMI per cow was calculated by dividing group total intake by the number of cows.

1.4.2 Nutrient Content in Feed, Feces, and Urine
Feed and fecal samples were dried at 65°C for 48 h, cooled for 24 h, and weighed to determine initial moisture. Two-gram samples were then oven-dried at 105°C for 3 h, cooled in a desiccator for 30 min, re-dried for 1 h, and re-weighed until the difference between two consecutive weights was <0.0002 g to determine adsorbed water content and calculate DM content. Daily DMI = average daily feed intake × DM content.

Crude fat (EE), calcium (Ca), and phosphorus (P) in feed and feces were determined according to national standards [13-15]. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were analyzed using an ANKOM-A2000i fiber analyzer (USA) per national standards [16-17]. Nitrogen content in feed, feces, and urine was determined by the Kjeldahl method using a FOSS-8400 analyzer (Denmark) per national standard [18]. Sulfur and molybdenum in feed were measured by ICP-MS (USA) per standards [19-20].

Apparent digestibility was calculated as:
Nutrient apparent digestibility (%) = 100 × (A - B) / A ,
where A is nutrient content in feed and B is nutrient content in feces.

1.4.3 Milk Composition
Milk fat, protein, lactose, solids-not-fat (SNF), total solids, and somatic cell count were determined using a MilkoScan™ Mars analyzer (Denmark).

1.4.4 Serum Biochemical Indices
Serum glucose (Glu) was measured by glucose oxidase method, total protein (TP) by biuret method, copper by complexometric colorimetry, and triglycerides (TG) by GPO-PAP method using a Microlab300 semi-automatic analyzer (Netherlands) according to kit instructions (Beijing Zhongsheng Beikong Biotechnology). Serum ceruloplasmin, malondialdehyde (MDA), triiodothyronine (T3), thyroxine (T4), immunoglobulin G (IgG), SOD, Cu-Zn SOD, GSH-Px activity, and total antioxidant capacity (T-AOC) were measured by ELISA using a Power Wave XS2 microplate reader (USA) per kit instructions.

1.4.5 Copper Content in Feed, Feces, Urine, and Milk
Copper content was determined by flame atomic absorption spectrometry (Zeenit-700P, Germany) according to national standard [21].

1.5 Statistical Analysis Data were analyzed by one-way ANOVA using SPSS 19.0. Duncan' s multiple range test was used for pairwise comparisons when significant differences were detected. Results are expressed as means ± SD, with $P<0.05$ considered significant and $P<0.01$ highly significant.

Results

2.1 Effects of Dietary Copper Levels on DMI and Performance As shown in , DMI did not differ among groups ($P>0.05$). Milk yield increased with dietary copper level, peaking in Group III, with a slight decline in Group IV. Groups II, III, and IV showed 4.91%, 6.27%, and 4.78% higher milk yield than Group I ($P<0.05$), though differences among supplemented groups were not significant ($P>0.05$).

Milk fat and protein percentages increased initially then decreased with copper level, with Group III showing 5.31% and 5.26% higher values than Group I, though differences were not significant ($P>0.05$). Dietary copper had no significant effects on lactose, SNF, or total solids ($P>0.05$).

Somatic cell count decreased with increasing copper, with reductions of 11.34%, 15.18%, and 17.42% in Groups II, III, and IV compared with Group I ($P<0.01$), but no differences among supplemented groups ($P>0.05$). Feed conversion ratio (FCR) tended to increase with copper level, with Groups II, III, and IV showing 4.26%, 5.67%, and 4.26% improvements over Group I, though not statistically significant ($P>0.05$).

2.2 Effects of Dietary Copper Levels on Nutrient Apparent Digestibility As shown in , apparent digestibility of CP, EE, Ca, and P did not differ among groups ($P>0.05$). NDF digestibility was 6.21% and 7.60% higher in Groups II and III than Group I ($P<0.05$), while ADF digestibility increased by 6.78% and 8.22% ($P<0.05$). No significant differences in NDF or ADF digestibility were observed between Groups I and IV ($P>0.05$).

2.3 Effects of Dietary Copper Levels on Serum Antioxidant Indices As shown in , serum copper concentration increased with dietary copper level. Compared with Group I, serum copper increased by 26.85%, 24.92%, and 41.03% in Groups II, III, and IV ($P<0.01$), with Group IV being significantly higher than other groups ($P<0.01$) but no difference between Groups II and III ($P>0.05$). Serum ceruloplasmin and activities of SOD, Cu-Zn SOD, and GSH-Px increased gradually with copper level. Ceruloplasmin increased by 9.93%, 20.71%, and 40.24% in Groups II, III, and IV ($P<0.01$), with all antioxidant enzyme activities being significantly higher than Group I ($P<0.01$). T-AOC increased initially then decreased, being significantly higher in Groups II, III, and IV compared with Group I ($P<0.01$ or $P<0.05$). Serum MDA decreased by 50.00%, 60.23%, and 47.08% in Groups II, III, and IV ($P<0.01$).

2.4 Effects of Dietary Copper Levels on Serum Biochemical Indices As shown in , serum TP increased gradually with copper level, with Groups II, III, and IV showing 2.48% ($P>0.05$), 6.45% ($P<0.01$), and 11.46% ($P<0.01$) increases over Group I. Serum Glu increased initially then decreased, with Groups II, III, and IV showing 10.25% ($P>0.05$), 25.09% ($P<0.01$), and 15.55% ($P<0.05$) increases. Serum TG did not differ among groups ($P>0.05$). Serum T3 and T4 increased with copper level, with Groups II, III, and IV showing 15.02%, 19.48%, and 36.29% increases in T3 ($P<0.01$) and 15.03%, 17.31%, and 33.98% increases in T4 ($P<0.01$). Serum IgG increased initially then decreased, with Groups II, III, and IV showing 49.56%, 165.56%, and 90.22% increases ($P<0.01$), with Group III being significantly higher than other groups ($P<0.01$).

2.5 Effects of Dietary Copper Levels on Copper Metabolism As shown in , daily fecal copper excretion increased with dietary copper level. Compared with Group I, fecal copper increased by 125.15%, 187.79%, and 250.83% in Groups II, III, and IV ($P<0.01$), with highly significant differences among all groups ($P<0.01$). Urinary copper increased by 6.32% ($P>0.05$), 40.27% ($P<0.05$), and 65.61% ($P<0.01$), respectively. Milk copper content was not

affected (P>0.05). Copper apparent digestibility did not differ among groups (P>0.05). Daily copper deposition increased with dietary copper, being significantly higher in Groups II, III, and IV than Group I (P<0.01), with Group III higher than Group II (P<0.05) and Group IV higher than both Groups II (P<0.01) and III (P<0.05). Copper deposition rate did not differ among supplemented groups (P>0.05) but was 80.37%, 78.76%, and 79.91% higher than Group I (P<0.01).

Discussion

3.1 Effects on DMI and Performance Dry matter intake is influenced by feed palatability, rumen fill, digesta passage rate, and cow metabolism [22-23]. Ward et al. [24] reported that copper supplementation increased DMI in cattle, but this study found no significant effect, possibly due to differences in breed, diet composition, or copper deficiency status. Milk yield is a key economic indicator. Supplementing 7.6 mg/kg copper increased milk yield by 4.85–9.61% without affecting milk fat [25-26]. In Holstein cows 100–120 days postpartum, copper supplementation increased milk yield by 3.3% and reduced net energy for lactation per kg of 4% FCM by 8.6% after 120 days [27]. Our results showed that milk yield, milk fat, milk protein, and FCR improved with copper level, but milk yield declined when dietary copper reached 28.09 mg/kg DM (copper intake: 624.44 mg/d) compared with 23.09 mg/kg DM (514.21 mg/d), consistent with Liu [6]. This suggests that copper requirements are influenced by internal and external factors [28], and excessive copper may negatively affect performance.

3.2 Effects on Nutrient Apparent Digestibility Many studies have shown that increasing dietary copper significantly improves NDF and ADF digestibility [29-31], which our results confirm. At 18.09 mg/kg DM (403.59 mg/d intake) and 23.09 mg/kg DM (514.21 mg/d), NDF and ADF digestibility increased significantly, possibly because adequate copper inhibits pathogenic bacteria and promotes beneficial microbial growth [31], or slows intestinal mucosal cell turnover, reducing energy requirements for maintenance [32]. Liu [6] reported maximal CP digestibility at 24 mg/kg copper supplementation, but our study did not observe this at 28.09 mg/kg DM, likely due to differences in diet composition [33].

3.3 Effects on Serum Antioxidant Indices Serum copper reflects copper metabolic status [34-35]. Normal serum copper in dairy cows ranges from 7.87 to 18.89 mol/L [36]. Suttle [37] indicated that plasma copper <9 mol/L indicates deficiency. Our results showed increasing serum copper with dietary level, consistent with Zhang [38]. Although all groups were within normal range, Group I (8.09 mg/kg DM, 179.84 mg/d intake) was at the lower end. Ceruloplasmin is a copper-containing glycoprotein that regulates copper distribution, synthesizes copper-dependent enzymes, and acts as an antioxidant with oxidase activity [39-40]. Our results confirm that ceruloplasmin increases with dietary copper [9].

Copper is essential for SOD, ceruloplasmin, and other enzymes, and deficiency adversely affects hemoglobin formation, bone development, and hair pigmentation [41]. Reactive oxygen species (O , ·OH, H O) produced during normal metabolism cause tissue damage [42]. SOD is the primary antioxidant enzyme that specifically scavenges O , forming the first line of antioxidant defense [43-44]. Copper deficiency reduces SOD activity and content, possibly by affecting SOD mRNA transcription or copper-dependent transcription factors (ACE1) [45]. Copper deficiency also decreases GSH-Px activity [46] by increasing O concentration, which inhibits GSH-Px [47]. T-AOC reflects overall antioxidant capacity, which is closely related to health status [48]. MDA, a major lipid peroxidation product, indicates cellular oxidative damage and free radical attack [49-50]. Our study confirmed that increasing dietary copper enhanced SOD, Cu-Zn SOD, GSH-Px activities and T-AOC while decreasing MDA, consistent with previous reports [2,51,52]. Normal serum MDA ranges from 1.20 to 2.23 mmol/L [53]; Group I (3.42 mmol/L) exceeded this range, while copper supplementation reduced MDA to normal levels. Yu et al. [54] reported that serum copper, ceruloplasmin, and Cu-Zn SOD plateaued at 31.5 mg/kg dietary copper. In our study, Group IV (28.09 mg/kg DM, 624.44 mg/d) showed the highest values, warranting further research on higher copper intakes. Overall, increasing dietary copper enhances antioxidant capacity to scavenge free radicals and lipid peroxides, though higher levels do not necessarily confer greater benefits.

3.4 Effects on Serum Biochemical Indices Serum TP, Glu, and TG reflect protein, carbohydrate, and lipid metabolism [55-57]. Normal serum Glu ranges from 2.3 to 4.1 mmol/L [58]. Our results showed increased TP, Glu (within normal range), and TG with copper supplementation, consistent with Wang et al. [51], indicating enhanced nutrient metabolism. T3 and T4 regulate basal metabolic rate, promoting glycogen, protein, and lipid catabolism for energy supply [59-60]. Copper deficiency reduces thyroid Cu-Zn SOD activity, increasing lipid peroxides that inhibit T3 and T4 synthesis, and decreases cytochrome oxidase activity, reducing ATP synthesis and inhibiting deiodinase activity, thereby blocking T3 production [61]. Normally, 40% of T4 is converted to the more biologically active T3 [62]. Our results showed increased T3 and T4 with copper supplementation, consistent with Qiao et al. [63] and Lukaski et al. [64], indicating improved thyroid function important for Glu utilization, lipid mobilization, lactation maintenance, and thermoregulation [65].

IgG plays a vital role in immunity. Copper deficiency reduces serum IgG [66-67], possibly by decreasing antibody numbers or immunoglobulin secretion [68]. Copper deficiency reduces lymphocyte cytochrome oxidase activity, decreasing ATP production and impairing IgG synthesis [69]. In chicks, high dietary copper decreased serum IgG [70], and both copper deficiency and excess reduced IgG in children [71]. Our study showed maximal IgG in Group III (23.09 mg/kg DM, 514.21 mg/d), with a decline in Group IV (28.09 mg/kg DM, 624.44 mg/d), possibly because high copper negatively affects immune organs, hindering B lymphocyte proliferation, differentiation, and maturation [72-75].

3.5 Effects on Copper Metabolism Copper is primarily excreted via the digestive tract, with biliary excretion being a major pathway for maintaining copper homeostasis; excretion is more important than absorption for regulating copper balance [76]. Dietary sulfur and molybdenum reduce copper utilization: sulfur is converted to sulfide in the rumen, forming insoluble copper sulfide [77], while sulfur and molybdenum form tetrathiomolybdate that binds copper, reducing absorption [78]. The effects depend on feed type: molybdenum has less effect on copper availability in silage, while sulfur markedly reduces it; molybdenum inhibits copper absorption from hay to a lesser extent; sulfur and molybdenum have greater effects on fresh forage than on hay or silage [8,79]. At low dietary molybdenum, added molybdenum strongly inhibits copper absorption, but this effect plateaus at 4–5 mg/kg DM [79].

Most supplemented trace elements are excreted in feces. Long-term application of manure as organic fertilizer can cause soil accumulation and crop yield reduction [80], while wastewater contamination reduces water self-purification capacity, causing aquatic plant death at 0.5 mg/kg copper [81]. Reducing supplementation decreases fecal trace element excretion [82-83]. Our results showed that fecal and urinary copper, milk copper, and copper deposition increased with dietary copper, while apparent digestibility remained unchanged. Although deposition rate was higher in supplemented groups than Group I (8.09 mg/kg DM, 179.84 mg/d), no differences were observed among Groups II, III, and IV, consistent with Li et al. [84]. According to Ministry of Agriculture Bulletin No. 2625 [85] and NRC (2001) [11], our dietary copper levels were below the maximum limit of 30 mg/kg DM and posed no toxicity risk. Fecal copper in Groups II and III was below China's 2008 Soil Environmental Quality Standard [86], posing no soil pollution risk. These results suggest that increasing dietary copper does not affect copper digestibility or deposition, possibly because our low-sulfur (0.29%) and low-molybdenum (1.96 mg/kg DM) diets, based primarily on corn silage, had minimal antagonistic effects on copper.

Conclusion

Under the conditions of this study, dietary copper supplementation effectively improved lactating performance, NDF apparent digestibility, antioxidant capacity, and immunity, but increased fecal and urinary copper excretion. Considering all factors, the optimal dietary copper level for dairy cows is 18.09–23.09 mg/kg DM (copper intake: 403.59–514.21 mg/d).

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