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Oxidative Stress and Imbalance of Mineral Metabolism Contribute to Lameness in Dairy Cows

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Abstract The objective of this study was to investigate correlations between oxidative stress, metabolism of mineral elements, and lameness in dairy cows. Forty multiparous Chinese Holstein dairy cows were selected and divided into two groups (healthy vs lame, $n=20$) by gait score. The experiment lasted for 60 days and samples of hair, blood, and hoof were collected at days 0, 30, and 60 of experiment period, individually. Compared with healthy cows, elevation of MDA, CTX-II, COMP levels, and GSSG/GSH ratio together with depletion of SOD and MT levels in the serum were revealed in lame cows. Simultaneously, significant decreased contents of Zn, Cu, and Mn in the serum, hair, and hoof samples were shown in lame cows, but there was no obvious difference in contents of P, Mg, and Ca (except hoof Ca) in the serum, hair, and hoof between healthy and lame cows. In addition, histological examination and the hardness test demonstrated a poor hoof quality in lame cows. In summary, oxidative stress is implicated in the pathogenesis of lameness caused by imbalance of nutrients (especially selective minerals promoting healthy hoof growth) in dairy cows.

Keywords Bone metabolic biomarker · Dairy cow · Lameness · Mineral element · Oxidative stress

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Introduction

Lameness is an important health problem on many modern dairy farms and has been ranked as the third most important health-related economic loss facing the dairy industry, following fertility and mastitis [1, 2]. It affects the welfare of dairy cows because lameness is associated with pain [3]. There is also a great economic loss for farmers, because lameness can result in decreased milk yield, reduced reproductive performance, high culling rates, and increased cost of veterinary intervention [4, 5]. The vast majority of lameness cases are caused by a variety of non-infectious and infectious hoof diseases.

It is important to note that causes of lameness in dairy cows are multifactorial. Factors affecting lameness and locomotion include laminitis or other inflammatory diseases of the hoof [6, 7], poor management (improper dairy stall design, slippery floor surfaces, and lack of exercise), and nutritional imbalances, etc. Recent studies have revealed that oxidative stress has been implicated in the pathogenesis of lameness [8–10]. Under oxidative stress conditions, excessive reactive oxygen species (ROS) can induce dyskeratosis of hoof tissue [11], chondrocyte apoptosis, and cartilage degeneration [9]. However, it is well known that trace elements have an important role in scavenging free radicals at the cellular level and hence could influence the anti-oxidant/free radical balance. What is more, adequate trace element nutrition plays a significant role in the production of quality hoof formation, which in turn has a positive improvement in lameness in lactating dairy cows [11]. For example, Zn, Cu, and Mn are involved in numerous biological pathways during horn production, while Ca, Mg, and P can improve hardness and density of hoof by speeding formation and regeneration [12, 13].

The most important way to balance oxidative damage and anti-oxidant defence in dairy cows is to optimize the dietary intake of anti-oxidant minerals. But, first, we must determine

the changes of related minerals in the body of lame cows. Therefore, the present study was conducted to determine the status of the six different minerals (Zn, Cu, Mn, Ca, Mg, and P) in three different tissues (serum, hair, and hoof keratin) and then compare their metabolisms between healthy and lame cows. In addition, this paper will address the changes in biomarkers of joint damage, hoof hardness, and morphology of hoof keratin between healthy and lame cows, which may provide a positive suggestion for prevention of cattle lameness.

Materials and Methods

Animals and Experimental Design

This study was conducted at a commercial dairy farm (Qingdao, China) from June 1, 2013 to July 30, 2013. All experiments were reviewed and approved by the Animal Care and Use Committee of Shandong Agricultural University (approval number 2013–05; 15 May 2013). The gait scores of all cows in this farm (n=927) were assessed using a five-point locomotion scoring system (Table 1) [14, 15] and the incidence of lameness is about 21 %. This study concentrated on lameness caused by nutritional factors, so cows that suffered from foot rot, sole ulcer, white line disease, solar abscess, and interdigital dermatitis were not included in the experimental design. Finally, forty multiparous Chinese Holstein dairy cows were selected with similar milk production, parity, and lactation and then assigned into two groups for healthy (gait scores as 1 and 2; n=20) and lame (gait scores as 3, 4, and 5; n=20) cows by gait score. All cows in this experiment were fed a total mixed ration containing corn silage, brewers grain, alfalfa hay, soy hulls, pasture hay, corn grain, wheat bran, soybean meal, cotton seed meal, limestone, dicalcium phosphate, saleratus, sodium chloride, magnesium oxide, and premix (Tables 2 and 3). Zn, Cu, and Mn sulfates were used in the premix. The experiment lasted for 60 days.

Table 1 Locomotion scoring system

Score	Description	Gait characteristics
1	Sound	Flat back, smooth head wobble frequency, joint activities with ease, uniform stride
2	Imperfect gait	Slightly arched back, smooth head wobble frequency, mild joint stiffness, slightly uneven gait, no visible lameness
3	Slight limp	Arched back, uneven head bobs, joint stiffness, uneven gait, slightly lameness
4	Noticeable limp	Obvious arched back, obvious head bobs, joint stiffness, hesitant gait, obvious lameness
5	Obvious limp	Severely arched back, very obvious head bobs, joint stiffness, difficulty walking, severely lameness

Collection of Tissues

Blood samples were collected from the coccygeal vein at days 0, 30, and 60 of experiment period and then centrifuged at 2000×g for 15 min to obtain the serum. Serum was used to determine assays of oxidative stress, biomarkers of joint damage, and mineral levels immediately. Samples of hoof stratum corneum were taken from the bulbar zone in the lateral claw of the right hindlimb at days 0 and 60 of experiment period. All hoof samples were stored in small sealed plastic containers to prevent dehydration and then further refined with a planning machine (with a slowly moving chisel). This equipment was used to make a sample with smooth, parallel surfaces and with precise dimensions. This procedure was requisite because all samples needed to have the same cross section for bending tests and the same surface area for compression tests. One part of hoof samples was fixed for morphological studies as later described, while another portion was used for the determination of hardness and mineral levels immediately. Hair samples were collected from tail for mineral element analysis at days 0 and 60 of experiment period.

Determination of Biomarkers of Oxidative Stress and Joint Damage

The levels of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT), malondialdehyde (MDA), reduced glutathione (GSH), oxidized glutathione (GSSG), and metallothionein (MT) in the serum were measured spectrophotometrically, in accordance with the analysis kit detection protocol (Jiancheng Bioengineering Institute, Nanjing,

Table 2 Ingredient compositions of the basal diet (%, DM basis)

Item	Ration
Corn silage	18.70
Brewers grain	6.92
Alfalfa hay	13.93
Soy hulls	3.30
Pasture hay	12.08
Corn grain	23.22
Wheat bran	7.37
Soybean meal	7.70
Cotton seed meal	4.28
Limestone	0.45
Dicalcium phosphate	0.45
Saleratus	0.23
Sodium chloride	0.68
Magnesium oxide	0.14
Premix[a]	0.55

[a] Formulated to provide (per kg of DM) 700,000 IU of vitamin A, 200,000 IU of vitamin D, 5,000 IU of vitamin E, 8,000 mg of Zn, 55 mg of Se, 20 mg of Co, 110 mg of I, 2,400 mg of Fe, 2,600 mg of Mn, and 2000 mg of Cu

Table 3 Chemical compositions of the basal diet (%, DM)

Item	Ration
Nutritional, % of DM	
CP	16.45
NDF	34.43
ADF	20.86
Ash	7.55
EE	4.8
Ca	0.91
P	0.46
Mg	0.24
Trace mineral (mg/kg)	
Zn	88.1
Cu	24.2
Mn	55.4

China). GSSG/GSH was calculated according to the levels of GSSG and GSH. Biomarkers of joint damage, including C-terminal telopeptide of type II collagen (CTX-II), procollagen-II N-terminal peptide (PIIANP), and cartilage oligomeric matrix protein (COMP), were measured by enzyme immunoassay kits (Lengton Inc., Shanghai, China).

Assessment of Minerals in the Serum, Hair, and Hoof

The collected serum was digested using HNO_3 and H_2O_2 (guaranteed reagents) in Teflon PFA microwave digestion vessels. Samples of hoof and hair were dried completely in an oven at 105 °C until their mass was constant. Then, the dried samples were weighted and digested using HNO_3 and H_2O_2 in Teflon PFA microwave digestion vessels. All digested samples were diluted with de-ionized water so that the analyte was within the calibration range. Concentrations of Zn, Cu, Mn, Mg, and Ca were determined by ICP-MS method according to the manufacturer's recommendation. Quality control was strictly carried out using standard reference materials (SRM 1,598, NIST). Concentrations of P in the serum, hair, and hoof were assessed by phosphomolybdate method with a commercial kit (Jiancheng Bioengineering Institute, Nanjing, China).

Hoof Quality Evaluation

Hoof quality was assessed by hardness test and histological examination of hoof keratin, individually. Hoof hardness was tested using a durometer (Shore D Scale) by ball indentation hardness [16]. Collected hoof samples were fixed in 10 % formalin solution for 1 week, softened in dilute nitric acid, dehydrated in ethanol and xylene, and embedded in paraffin. Sections of 5 μm thick were cut and stained with hematoxylin and eosin (HE) for histological assessment. The slides were examined under a light microscope (Nikon Eclipse E600W) by the same pathologist blinded to the treatment.

Statistical Analysis

The analysis of all data was performed using PROC ANOVA of SAS (SAS Inst. Inc., Cary, NC) with cows as the experimental unit and treatment as the only effect in the model. Data were expressed as means±SEM. Significant differences were declared at $P \leq 0.05$, and tends were reported at $0.05 < P \leq 0.10$.

Results

Analysis of Oxidative Stress Status in Lame Cows

Information of oxidative stress and antioxidant defence system in the serum is shown in Table 4. MDA level is commonly known as a marker of oxidative stress, while the GSSG/GSH ratio is another useful indicator of oxidative stress in the body. Compared with healthy cows, increased MDA level and GSSG/GSH ratio were observed in lame cows, demonstrating that oxidative stress has been implicated in the pathogenesis of lameness. There is no significant difference in activities of GSH-Px and CAT and levels of GSH and GSSG ($P>0.1$), but SOD activity and MT level showed a significant decrease ($P=0.045$ and 0.017, respectively) compared with the control group. The above results indicate that insufficient levels of antioxidants (enzymatic and non-enzymatic) may be related to oxidative stress status in lame cows.

Table 4 Levels of oxidative stress markers and antioxidants in the serum of dairy cows

Item	Treatment		*P* value
	Healthy cows	Lame cows	
MDA (nmol/mL)	5.40±0.32	6.36±0.23**	0.018
SOD (U/mL)	55.04±2.02	50.81±1.16**	0.045
CAT (U/mL)	5.32±0.62	5.11±0.71	0.825
GSH-Px (U/mL)	75.63±6.15	73.56±7.56	0.561
MT (ng/mL)	25.05±2.03	20.55±2.28**	0.017
GSH (mg/L)	7.16±0.69	6.33±0.53	0.155
GSSG (μmol/L)	284.06±25.41	299.33±20.99	0.179
GSSG/GSH	0.0402±0.005	0.0466±0.003**	0.049

Data are shown as means±SEM ($n=20$). Levels of significance are set at *$P \leq 0.10$, ** $P \leq 0.05$

Distribution of Six Mineral Elements in Serum, Hair, and Hoof Keratin

Mineral elements are essential cofactors for multiple enzymes and have an important role in maintaining redox homeostasis and normal development of claw horn. As for the hoof keratin formation, complex biochemical procedures take place during the keratinization process. Among the minerals, the following mineral materials (Zn, Cu, Mn, Ca, Mg, and P) are necessary. As shown in Tables 5 and 6, decreased levels of Zn, Cu, and Mn were detected in the serum and hair of lame cows, whereas there is no significant difference in the distribution of macroelements (Ca, Mg, P) between healthy and lame cows. Furthermore, mineral composition of lame cow's hoof keratin is different from that of healthy cow (Table 7), i.e., lame cows have significant lower levels of Zn, Cu, Mn, and Ca than healthy cows, but no significant difference was observed on contents of Mg and P.

Evaluation of Joint Damage and Hoof Quality in Lame Cows

Lameness is commonly accompanied by joint damage. Among the joint tissues, cartilage is most susceptible to damage, while it is mainly composed of collagen type II (70 %) and its homeostasis consists in balance between degradation and formation. Therefore, we focused on three main cartilage biomarkers (CTX-II, COMP, and PIIANP) to analyze cartilage breakdown and formation in lame cows (Table 8). Compared with healthy cows, increased serum COMP level was observed in lame cows (P=0.036). Moreover, serum CTX-II level tended to be higher in lame cows than that in healthy cows (P=0.081). But, there was no significant difference in serum PIIANP level between healthy and lame cows (P>0.1).

In addition, hoof quality was assessed by hoof hardness and histologic examination, respectively. It showed that lame cows had significantly decreased hoof hardness than healthy cows (Table 8). Meanwhile, hoof stratum corneum of dairy cows was collected to make the pathologic assessment by microscopic techniques (Fig. 1). Microscopic appearance of healthy cows showed normal structure of collagen fibers with more dense and compact staining (Fig. 1a, c). Irregular arrangement of collagen marked by enlarged structure (loop in the figure) with fragmentation of collagen fibers as well as reduced staining density (decreased collagen contents) was evident in samples of all lame cows (Fig. 1b, d). It is noteworthy that there are some horn tubules with a concentric pattern around the lumen in the samples of lame cows, which are normal structure of bulbar horn [17].

Table 5 Changes in serum mineral levels between healthy and lame cows

Item	Treatment		P value
	Healthy cows	Lame cows	
Zn (mg/L)	1.93±0.09	1.75±0.08**	0.032
Cu (mg/L)	1.02±0.02	0.95±0.03**	0.008
Mn (μg/L)	77.08±4.07	71.58±2.02**	0.045
Ca (mg/L)	93.60±1.49	91.93±2.55	0.552
Mg (mg/L)	39.98±1.79	38.16±1.20	0.140
P (mg/L)	124.93±3.58	127.75±3.43	0.438

Values are shown as means±SEM (n=20). Levels of significance are set at *P≤0.10, ** P≤0.05

Table 6 Comparison of six hair minerals contents in healthy and lame cows

Item	Treatment		P value
	Healthy cows	Lame cows	
Zn (mg/kg)	70.83±0.88	67.90±0.86**	0.014
Cu (mg/kg)	14.43±1.06	11.51±0.51**	0.019
Mn (mg/kg)	13.49±0.46	10.57±0.66**	0.009
Ca (mg/kg)	1.86±0.23	1.80±0.14	0.769
Mg (mg/kg)	0.88±0.11	0.84±0.08	0.629
P (g/kg)	0.45±0.04	0.43±0.09	0.413

Values are shown as means±SEM (n=20). Levels of significance are set at *P≤0.10, ** P≤0.05

Discussion

Hoof diseases can be caused by many factors and lameness is the ultimate manifestation of a variety of conditions that may have distinctly different origins [11]. It is known that oxidative stress is involved in the pathogenesis of lameness in dairy cows [18]. Moreover, mineral elements play an important role in oxidative stress status and hoof development. Herein, this study was designed to investigate the lameness related to nutrition factors (animals were chose to satisfy the

Table 7 Levels of six mineral elements in hoof keratin of healthy and lame cows

Item	Treatment		P value
	Healthy cows	Lame cows	
Zn (mg/kg)	58.76±1.64	54.59±1.05**	0.037
Cu (mg/kg)	9.73±0.76	7.48±0.42**	0.042
Mn (mg/kg)	6.01±0.25	5.00±0.43**	0.012
Ca (g/kg)	1.24±0.13	1.01±0.09**	0.045
Mg (g/kg)	0.69±0.04	0.65±0.03	0.186
P (g/kg)	0.27±0.01	0.25±0.01	0.528

Data are shown as means±SEM (n=20). Levels of significance are set at *P≤0.10, ** P≤0.05

Table 8 Serum biomarkers of joint damage and hoof hardness in healthy and lame cows

Item	Treatment		*P* value
	Healthy cows	Lame cows	
CTX-II (ng/mL)	104.09±5.03	112.86±4.19*	0.081
PIIANP (ng/mL)	30.54±1.86	28.98±1.88	0.163
COMP (ng/mL)	59.98±3.07	68.16±4.31**	0.036
Hardness (HD)	30.2±0.6	27.7±0.5**	0.009

Values are shown as means±SEM (n=20). Levels of significance are set at *P≤0.10, ** P≤0.05

experimental requirement as described above), focusing on the relationship between oxidative stress, changes in mineral metabolism in different tissues, and hoof quality.

Under the same basal diet and feeding management, increased serum MDA level and GSSG/GSH ratio in lame cows further verified that oxidative stress is involved in lameness in cows. Furthermore, significantly decreased levels of enzymatic antioxidant (SOD) and non-enzymatic antioxidant (MT) were shown in lame cows, which provided another strong evidence for the potential role of oxidative damage in the pathogenesis of lameness. Trace elements have a specific role in free radical control at the cellular level and influence the anti-oxidant/free radical balance [19]. Among these microelements, Zn, Cu, and Mn are essential components of certain endogenous antioxidants. For example, Zn is the key factor to maintain an adequate level of MT (one potent free radical scavenger) and an essential component of SOD [20]. As an integral part of SOD, Cu and Mn take part in its anti-oxidant defense [19]. Given the special role of these minerals in anti-oxidant activities, this study was undertaken to determine the metabolic status of Zn, Cu, and Mn in dairy cows. Mineral elements levels in blood fluctuate rapidly in response to changing physiological and/or environmental conditions and therefore reflect real-time metabolic state of mineral elements [21]. Herein, serum mineral levels in this study were determined at three different time points (0, 30, and 60 days) and then averaged to make the data more accurately and convinced. Whereas, hair can provide a more permanent record of mineral elements associated with normal and abnormal metabolism [21]. As shown in Tables 5 and 6, metabolic disorder of Zn, Cu, and Mn occurred in lame cows. Based on these results, metabolic abnormalities of Zn, Cu, and Mn promoted the lameness in dairy cows, which were intimately related with enhanced oxidative stress.

In addition to their role in reducing oxidative stress, Zn, Cu, and Mn are essential nutrients to produce and maintain healthy hoof tissues [11]. Zn has been identified as a key mineral in the processes of horn production (keratinization) and plays a role in formation of structural keratin proteins, while insufficient supplies of bioavailable Zn may predispose cows to production of inferior horn tissue [12]. Cu is the key element to activate thiol oxidase enzyme which is responsible for formation of the chemical bonds between keratin filaments [22]. This process is essential for structural strength on the cellular level giving rigidity to the keratinized cell matrix [12]. Cattle

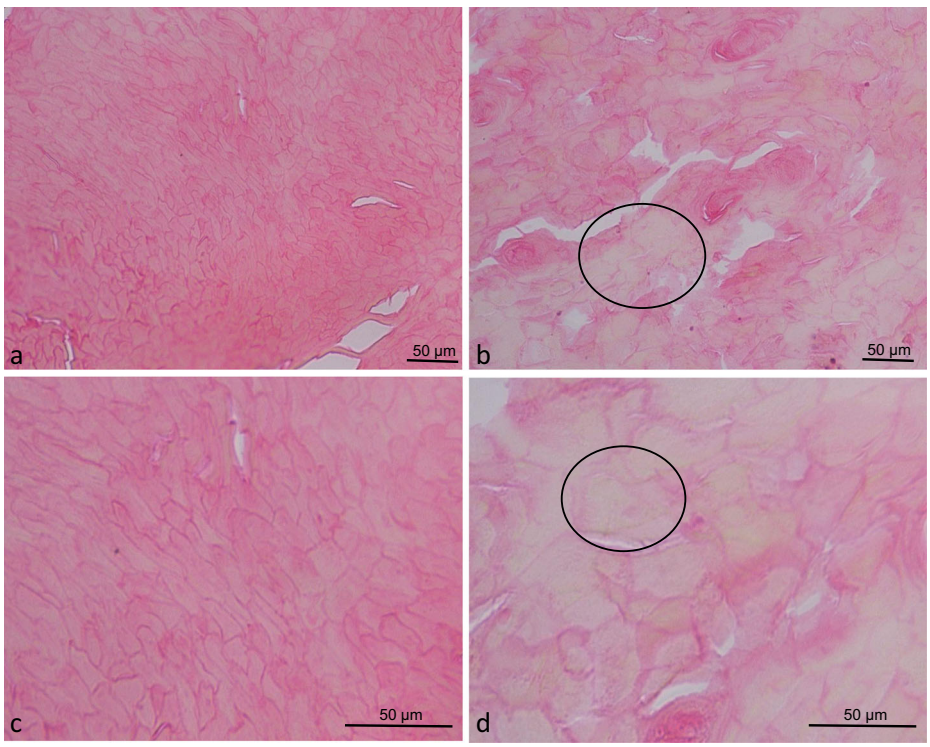

Fig. 1 Representative light microscopic appearance of hoof tissues in dairy cows. Healthy cows (**a**, **c**) with normal structure of collagen fibers in hoof stratum corneum. Lame cows (**b**, **d**) with damaged structure of collagen fibers. Pathological changes are marked in the figures

suffering from a subclinical copper deficiency are more susceptible to hood diseases [23]. Mn plays an indirect role in the keratinization process, and it is needed for activation of galactotransferase and glycosyltransferase enzymes, which are needed for the synthesis of chondroitin sulfate side chains of proteoglycan molecules [24, 25]. Proteoglycans are essential building blocks in formation of normal cartilage and bone. Combined with results in this study, lame cows exhibited decreased levels of hoof Zn, Cu, and Mn, which may be induced by insufficient storage of Zn, Cu, and Mn in the body. Furthermore, other minerals take part in horn production. Ca plays an integral role in the keratinization and cornification process and it is essential for the final steps in the production of the mature horn cell [26]. Insufficient Ca provided to maturing keratinocytes may lead to formation of dyskeratotic horn. P and Mg are also integral to build healthy hoof horn and bones. Firstly, P and Ca are bound by a specific ratio for proper hoof growth, while poor Ca-to-P ratios can cause brittle or tender hooves. Secondly, Mg helps with circulation and Ca absorption and its deficiency may directly result in a decrease in bone formation and affect bone stiffness [27]. As shown in this study, poor hoof quality in lame cows may be induced by deficiencies of Zn, Cu, and Mn and exhibited by decreased hoof hardness (Table 8), fragmentation, and disorganization of collagen fibers with decreased collagen contents (Fig. 1). Moreover, macroelement (Ca, P, and Mg) metabolism did not show abnormality in the serum, hair, and hoof (except lower hoof Ca levels), which may be related to their bioavailability. Lower hoof Ca level was connected with hoof hardness in lame cows, and the reason of its decrease will be worthy of further investigation.

Additionally, joint damage is the most common cause of lameness. Degeneration of cartilage can cause chronic inflammation in the joint, while this inflammation can further break down the cartilage. Type II collagen is the main constituent of cartilage matrix. As a sensitive and specific index of type II collagen degradation, CTX-II adequately reflects cartilage destruction [28]. Serum COMP is another marker that indicates increased cartilage degradation in arthritis [29]. And, PIIANP can be used as one specific marker of type IIA procollagen synthesis in articular diseases [30]. Therefore, increased serum CTX-II and COMP levels (Table 8) in lame cows verified that increased cartilage breakdown is involved in lameness of dairy cows. Cartilage breakdown in lame cows may be due to disorders of Zn, Cu, and Mn metabolism because they are intimately linked to cartilage growth and collagen synthesis [31]. Zn plays an important role in the normal growth and remodeling of articular cartilage [32]. Cu is required as a cofactor for lysyl oxidase which is essential for cartilage synthesis [33]. Mn is necessary for the synthesis of chondroitin sulfate [34]. Moreover, oxidative stress may be another factor that promotes cartilage destruction [35]. Based on these results, we speculate that metabolic imbalances of trace element (Zn, Cu, and Mn) and excessive oxidative stress result in abnormal cartilage development.

In summary, oxidative stress plays a critical role in lameness of dairy cows induced by disorders of mineral metabolism. Among these minerals related to hoof quality, inadequate Zn, Cu, and Mn promoted oxidative damage, increased cartilage breakdown and poor hoof quality (hoof hardness and histological assessment). However, macroelement (P, Mg, and Ca, except hoof Ca) metabolism has no evident association with lameness in this experiment. This paper will provide a positive suggestion for dairy farms with similar diet ingredients and lameness, but the detailed mechanism of lameness and mineral metabolism remains to be further investigated.

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