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# Varying Dietary Levels of Molybdenum Inducing Cell Apoptosis of Spleen Under Cadmium Stress in Caprine

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**Abstract** The present experiment aims at evaluating chronic toxic effects of the combination of cadmium (Cd) and molybdenum (Mo) according to residual element contents, apoptosis gene expression, and ultrastructure and histopathology changes of caprine spleen. In total, 36 Boer *goats* were randomly divided into four groups with the equal number in each group. The control group was orally administered with deionized water while the experimental groups I, II, and III were administered with the equal quantity of $CdCl_2$ (1 mg $kg^{-1}$ BW) and $(NH4)_6{\cdot}Mo_7O_{24}{\cdot}4H_2O$ including 15, 30, and 45 mg·Mo $kg^{-1}$ BW, respectively. Three individuals from each group were treated with euthanasia on days 0, 25, and 50. The data showed that the content of splenic residual Mo and Cd increased ($P < 0.05$) in the experimental groups on days 25 and 50, while no significant difference was observed in the content of Cu. The apoptosis-related gene expression levels including *Bcl-2*, *Bax*, *Caspase-3*, *Smac*, and *ceruloplasmin* (*CP*) were also determined. Results showed that significant reductions were observed in *Bcl-2* and *CP* expressions ($P < 0.01$), while *Caspase-3* gene was up-regulated ($P < 0.05$). However, no significant difference was observed in *Smac* and *Bax* expressions. Furthermore, on day 50, spleen tissues were presented to observe ultrastructural changes in lesions by means of transmission electron microscopy, with fragmentized nucleus, vesiculation of cytoplasm, mitochondria hyperplasia, and increasing lysosomes included. In addition, histopathology results corroborated the toxicity by showing cell hemorrhage, thickening central arteries, and enhanced capsule thickness. To sum up, our study revealed that the combination of Cd and Mo could induce remarkable damage to the spleen of *goats* by promoting cell apoptosis in the mitochondrial pathway and affecting the deposition of Mo and Cd.

**Keywords** Molybdenum · Cadmium · Spleen · Apoptosis · Caprine

## Introduction

Molybdenum (Mo) is a metal of vital industrial importance and is extensively applied in the steel industry. Moreover, Mo is an essential trace element for many animals and plants and has already been confirmed as a critical component for xanthine oxidase, aldehyde, and sulfite oxidase [1]. However, excessive dietary Mo intake turned out to be toxic for many species including rabbits, mice, and sheep [2–4]. In 1981, high-level Mo in feeding stuff was first discovered to be responsible for the persistent diarrhea in cattle, with features such as red skin and white hair, in southern Jiangxi Province, China [5]. Mo is traditionally regarded as playing a causative role in the secondary deficiency of copper and being poisonous when transformed to thiomolybdates [6, 7]. In

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addition, an overdose of Mo intake has been reported to result in kidney and liver damage by means of cell apoptosis [8, 9].

Cadmium (Cd) is an environmental toxicant worldwide, with its existence primarily resulting from industrial and agricultural emissions [10]. Chronic Cd accumulation in vivo leads to liver and spleen disorders [11]. Cd-induced reactive oxygen species could trigger oxidative stress damage, and the latter could impair spleen immune function by causing splenocyte apoptosis [12]. Also, some findings confirmed that mitochondrial pathway activation was another possible means inducing apoptosis in Cd toxicity [13, 14].

Because of the higher-than-permitted plant emission and surplus mining industry [15], livestock's Mo and Cd exposure was more common in Jiangxi Province than in other parts of China, and the mixed intoxication of Mo and Cd occurred more frequently in Jiangxi, China. However, the relationship between these two elements remains a controversy at the present time. On the one hand, Mo may provide protection against the highly concentrated heavy metal (e.g., Cd) toxicity [16] whose mechanism is somehow linked to Cd-metallothionein induction enhancement [17]. A combination of Mo and sulfur in feeding stuff has showed its efficiency on reducing Cd accumulation in sheep tissues [18]. On the other hand, some previous studies reported that Mo and Cd might have a synergistic effect on kidney and testicle tissues [19, 20]. The objective of the current study was to evaluate the effects of the combination of Mo and higher level of Cd on *caprine* spleen and to explore the mechanism relationship between the two elements by studying apoptosis-related genes on mRNA levels and histopathological changes in vivo.

## Materials and Methods

### Animals and Treatments

Thirty-six clinically healthy Boer *goats* aging between 5 and 6 months and weighing from 15 to 20 kg were purchased from an intensively commercial farm with vaccination and deinsectization in advance. The *goats* were randomly divided into four groups with each group containing nine animals. They were housed for 2 weeks under sanitary conditions for acclimation before the experiment commencement and provided with feed and water ad libitum. *Goats* in the control group were orally administered with corresponding quantitative deionized water, while *goats* in the treatment groups were orally administered with identical levels of $CdCl_2$ (Cd 1 mg $kg^{-1}$ BW) and varying doses of ammonium molybdate [$(NH_4)_6{\cdot}Mo_7O_{24}{\cdot}4H_2O$] (Mo 15 mg $kg^{-1}$ for group I, 30 mg $kg^{-1}$ for group II, and 45 mg $kg^{-1}$ for group III). Basic diet for *goats* met the standard nutritional requirements for *goat* breeding set by the National Research Council [21]. The content of the basic diet and the content of Mo and Cd in grass, water, and fodder are listed in Tables 1 and 2. The whole experiment span was 50 days. All animal care and experimental procedures were approved by the institutional ethics committee, and this study also complied with the criteria in Guide for the Care and Use of Laboratory Animals.

### Sample Collection

A total of 12 *goats* were randomly selected from four groups (three individuals in each group). Before sampling, they were performed with euthanasia with an overdose intravenous injection of sodium pentobarbital (100 mg $kg^{-1}$, Nembutal, Abbot Labs, IL, USA), and subsequently, spleen tissues were collected instantly from 12 *goats* in vivo on days 0, 25, and 50, respectively.

### Splenic Mo, Cd, and Cu Level Determination

The trace elements including Mo, Cd, and copper (Cu) in spleen were measured by using an Agilent 240 AA atomic absorption spectrophotometer (Agilent, USA) after wet-ashing the samples. All analyses were carried out according to the manufacturer's instructions.

### RT-PCR for Determining Apoptosis-Related Genes Expression in Spleen

Total spleen RNA was extracted by using TRIzol reagent according to the manufacturer's instructions (Invitrogen, California, USA). The first-strand complementary DNA

**Table 1** Composition and nutrient levels in the basal diet for the *goats*

| Composition of diet | | Nutrient levels | |
|---|---|---|---|
| Ingredients | Content (%) | Index | Levels |
| Maize | 52.5 | CP (%) | 16.57 |
| Deoiled rice bran | 19.0 | ME (MJ $kg^{-1}$) | 13.12 |
| Soybean meal | 10.0 | Ca (%) | 0.90 |
| Rapeseed meal | 7.0 | P (%) | 0.78 |
| Cottonseed meal | 7.0 | | |
| $CaHPO_4$ | 1.0 | | |
| Limestone | 1.5 | | |
| Salt | 1.0 | | |
| Additives[a] | 1.0 | | |
| Total | 100 | | |

[a] Per kilogram of additives contained the following: nicotinic acid 2000 mg; VA 1,000,000 IU; VD 3,250,000 IU; VE 2400 mg; Fe ($FeSO_4{\cdot}H_2O$) 2000 mg; Zn ($ZnSO_4{\cdot}H_2O$) 140,000 mg; Mn ($MnSO_4{\cdot}H_2O$) 3000 mg; I (KI, 3 %) 180 mg; Se ($NaSe_3O4{\cdot}H_2O$) 100 mg

**Table 2** The content of Mo and Cd in water, grass, and fodder (μg/g)

| Items | Micronutrient levels | |
|---|---|---|
| | Mo | Cd |
| Deionized water | 0.0000 | 0.0000 |
| Tap water | 0.0089 | 0.0008 |
| Grass | 1.8888 | 0.0708 |
| Fodder | 6.0195 | 0.0496 |

(cDNA) was synthetized with Titanium® RT-PCR Kit (Clontech, CA, USA) by strictly following the manufacturer's instructions. Expression of apoptosis-related genes, including *Bax*, *Bcl-2* and *Caspase-3*, *Smac*, *Ceruloplasmin* (*CP*), and *β-actin*, was evaluated according to previously published RT-PCR assays [22]. Relative expression was calculated according to RT-PCR efficiency formula reported [23]. The reference genes (*β-actin*) worked as an internal control for normalization of the results.

### Histopathological Examination

The spleen samples were fixed in 10 % neutral-buffered formalin and embedded in paraffin. Sections with 5-μm thickness were incised from each block and stained with hematoxylin and eosin (H&E). After that, the optical microscope was used to observe the histopathological results.

### Transmission Electron Microscopy

Transmission electron microscopy (TEM) studies were performed based on the protocol as previously mentioned [24]. After removal of spleen samples, they were performed and observed under TEM Zeiss 900 (Zeiss, Germany). The ultrastructural pathological changes in splenocytes were compared with the counterparts of the control group.

### Statistical Analysis

The experiments were performed in triplicate, and data were analyzed with the software Microsoft Excel 2007, OriginPro 9 (OriginLab Corporation, Northampton, MA, USA), SDS 2.4, and SPSS version 17.0 (SPSS Inc., Chicago, IL, USA). All experimental data were tested by the analysis of variance with significant differences between means determined by Duncan post hoc tests. The data were represented as mean ± SD. Differences were considered significant. Statistical significance level was set at a $P$ value < 0.05.

## Results

### Mo, Cu, and Cd Content in Spleen

Splenic Mo, Cd, and Cu concentrations are shown in Fig. 1. Splenic Mo concentration was supposed to be positively connected with Mo's intake. Three experimental groups showed increases on days 25 and 50, and they were statistically different among each other as administered Mo concentration increased. Cd content was boosted significantly ($P < 0.05$) in all experimental groups on day 50. Surprisingly, Cu concentrations in the four groups were not significantly different on days 25 and 50.

### The Relative mRNA Expression of Apoptosis-Related Genes

The data are illustrated in Fig. 2. *Bcl-2* mRNA expression in group II significantly decreased ($P < 0.05$) on day 25 compared with that in the control group, and its expression reduced ($P < 0.01$) in all experimental groups on day 50. Furthermore, *CP* mRNA expression in spleen plunged on day 25 ($P < 0.05$) while that in all experimental groups significantly plummeted when compared with that in the control group on day 50 ($P < 0.05$). Interestingly, no significant difference was observed in the *Bax* and *Smac* expressions on day 25 and 50. Moreover, *Caspase-3* mRNA expression in groups I and III were found to have been increased ($P < 0.05$) on day 50.

### Histopathological Results

No abnormal architecture was found in spleens from the control group, including regular central artery and capsule thickness (Fig. 3a, b). Samples in group I showed slight spleen lesions, with evident hemorrhage spots among splenocytes (Fig. 3c, d). In group II, spleens showed more serious degree of hemorrhage and thickening central artery layer (Fig. 3e, f). In addition, samples in group III showed an increase of capsule thickness and the most serious hemorrhage degree (Fig. 3g, h).

### TEM Observation

Electron microscope revealed splenocytes' ultrastructural changes induced by toxic effects of different levels of Mo and a certain level of Cd. The spleen cell's integrative organelles in the control group are shown in Fig. 4a. On day 50, decreased organelle numbers, nucleus deformation, and some high-density electron bodies were observed in group I (Fig. 4b). Pathological lesions are more serious in group II fed by Cd and mediate level of Mo (30 mg $kg^{-1}$) with apoptosis phenomenon including nuclear deformation and the

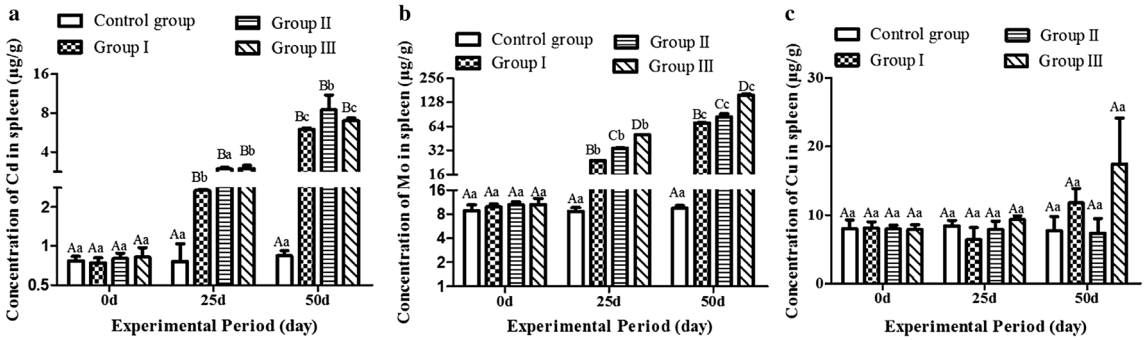


**Fig. 1** Residual content of Mo, Cd, and Cu in the splenocytes of *goats*. *Capital letters* stand for intra-group comparison and *small letters* stand for inter-group comparison. *Different letters* mean statistical difference ($P < 0.05$). It is the same below

vesiculation of mitochondrion whose cristaes and membranes also started to rupture and even dissolve (Fig. 4c). Figure 4d illustrated the effect of Cd with high level of Mo (45 mg $kg^{-1}$) on splenocyte, which demonstrated a more noticeable cell apoptosis with fragmentized nucleus and vesiculation of cytoplasma. In addition, some mitochondria dissolved to a large extent, and mitochondria hyperplasia and increasing numbers of lysosomes were also presented.

## Discussion

Affluent metal mineral resources in Jiangxi Province plus excessive mineral mining raised environmental problems and made livestock and birds under risky circumstances including Mo and Cd. High level of Mo in feeding stuff was first discovered to cause animals' persistent diarrhea, and "Red Skin and White Hair" syndromes in southern Jiangxi Province turned out to be a result of the combination of Mo and Cd rather than that of Mo alone [5], which made our research meaningful for local animal breeding. In the present study, residual element contents, apoptosis gene expression, and TEM were determined in order to evaluate the relationship between Mo and Cd on spleen cell apoptosis. Residual Mo and Cd concentration in spleen were positively linked with experimental time and administered dose. Besides, residual Cu concentration and *CP* expression were determined for splenic Cu metabolism evaluation. *CP* is a Cu-containing glycoprotein whose main function is to transfer Cu to the blood, which means Cu is mainly used in the body [25]. Therefore, *CP* expression is used as a reliable indicator of Cu nutrition status in vivo. In the current study, *CP* expression decreased as the experimental time lasted. This could potentially be explained by the fact that an overtake of Mo may bound Cu in

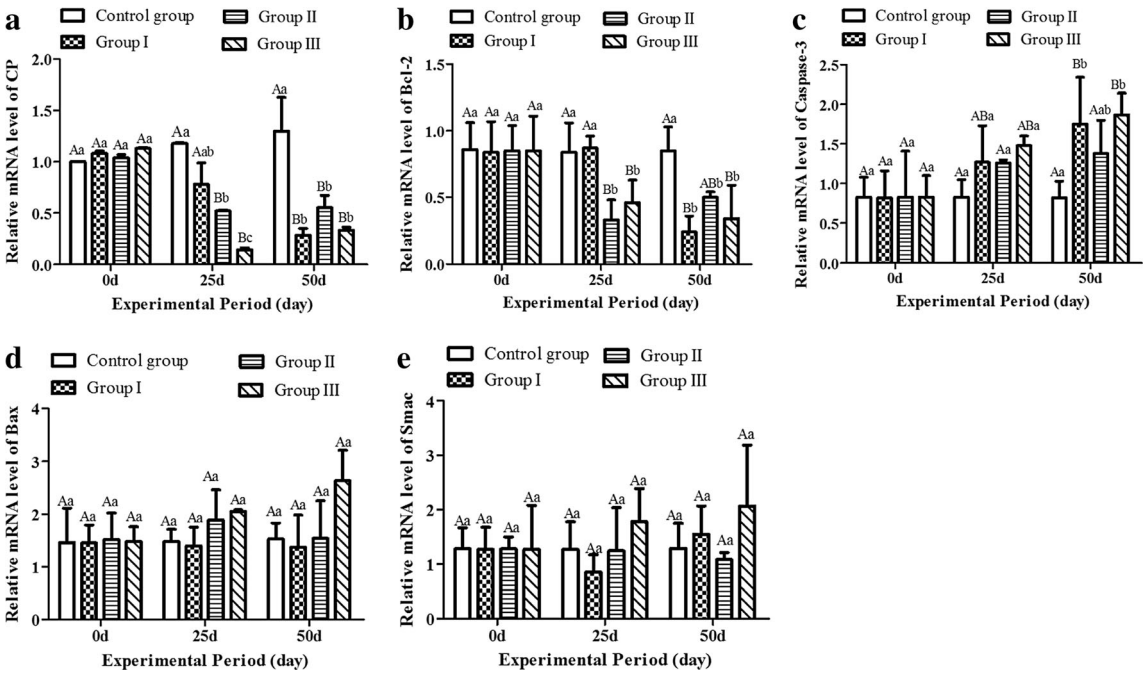


**Fig. 2** Determination of the mRNA expression of apoptosis-related genes in the *goat* spleen

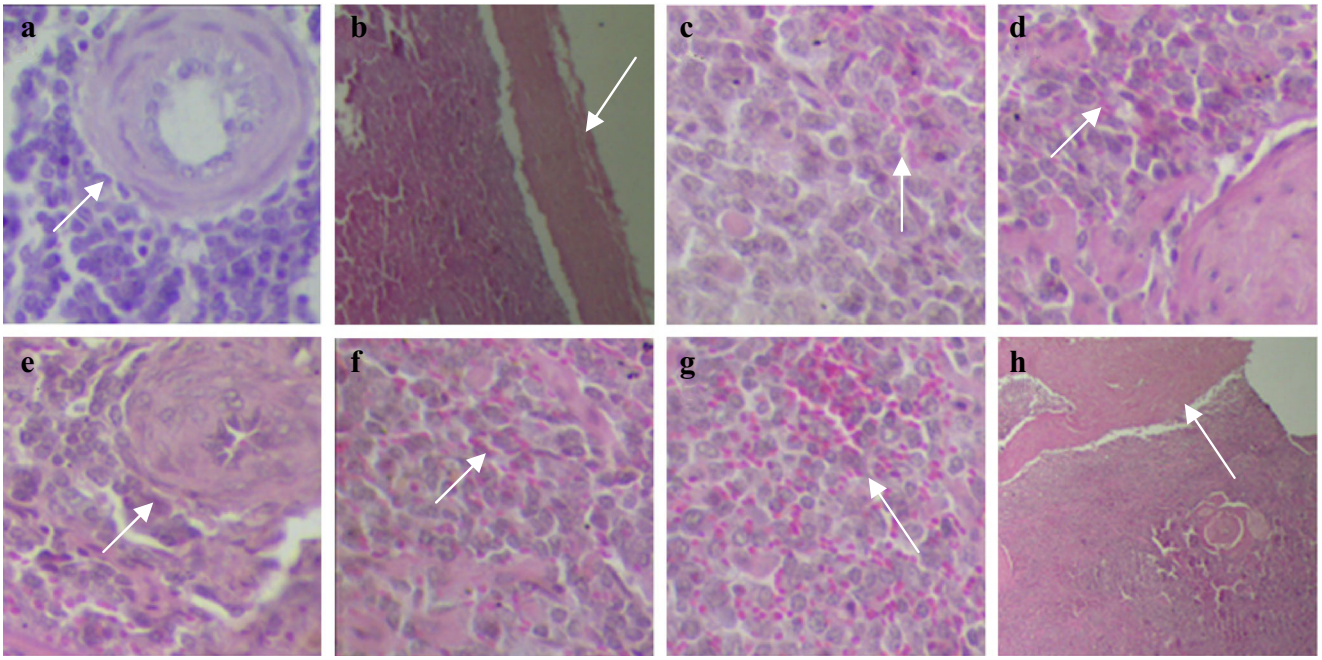


**Fig. 3** Histological results of the spleen in *goats* on day 50. Control group (**a**, **b**). Group I (**c**, **d**). Group II (**e**, **f**). Group III (**g**, **h**)

the Cu-Mo complex which is biologically unavailable in vivo [26], which shows available Cu absorption disturbance and Cu excretion disorder. However, the biological Cu-Mo antagonism was not self-evident according to the data. Thiomolybdates are supposed to form in the rumen by a reaction between sulfide and molybdate, binding with Cu or Cu proteins in the gastrointestinal tract and in the blood and tissues to render Cu unavailable for normal absorptive or

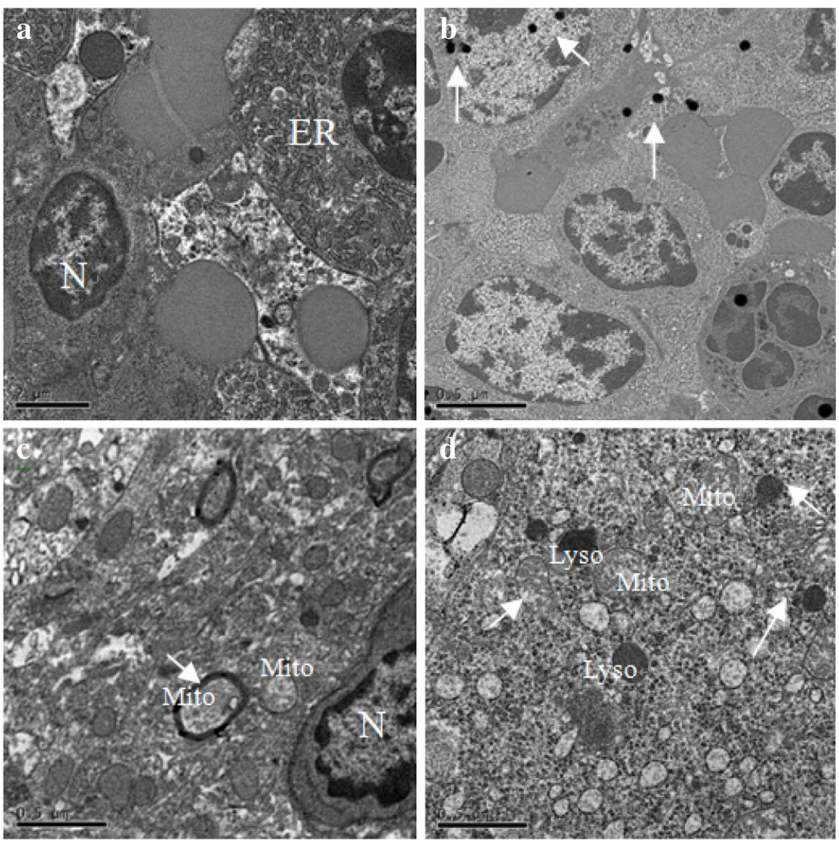


**Fig. 4** Ultrastructural images in the spleen cell of experimental *goats* on day 50. **a** Control group. **b** Group I.**c** Group II. **d** Group III.(Note: *N* nuclei, *Mito* mitochondria, *ER* endoplasmic reticulum, *Lyso* lysosome)

enzymic functions [27]. Cd was reported to promote the complex with metallothionein [28]. Cd-metallothionein was assumed to play a potentially causative role in removing Cu from the Cu-Mo-S complex and changing into absorbable forms [22]. The results suggested that the combination of Mo and Cd could possibly impair the splenocyte function of Cu's absorption and excretion; the data also indicated that the combination of Mo and Cd could initiate splenic antioxidant defense reduction and inhibit anti-oxidant indicators which also induce cell apoptosis.

Mitochondria play key roles in inducing apoptosis in ruminants' cells [29]. Mitochondrial malfunction is regarded as an early sign of cell apoptosis. Mitochondria-induced apoptosis begins with upstream *Caspase* activation and is regulated through members of the Bcl-2 family. *Bcl-2*, an oncogene belonging to the anti-apoptotic family, was expected to regulate cell apoptosis at mitochondrial level, suppressing apoptotic death [30]. However, suppression of prosurvival *Bcl-2* proteins is insufficient to kill cells in the absence of *Bax* [31]. *Bax*, a proapoptotic gene, was also believed to be involved in cell apoptosis by boosting the permeability of the outer mitochondrial membrane and promoting efflux of apoptotic proteins into the cytoplasm. The apoptotic cascade involving in the activation of *Caspase-3* was considered a downstream event in the mitochondrial pathway [32]. In the current study, the toxicity of a constant Cd with varying levels of Mo significantly down-regulated *Bcl-2* expression but promoted *Caspase-3* expression, with *Bax* and *Smac* remaining relatively unchanged. As for the proapoptotic gene, no remarkable difference was observed among the four groups in *Bax*, while *Bcl-2* decreased significantly. This is partly different from some previous reports saying dietary Mo could enhance *Bax* protein and decrease *Bcl-2* protein presence [33]. However, this could be explicable if the ratio of *Bcl-2*/*Bax* constitutes a rheostat model for apoptosis susceptibility evaluation [34]. The inhibition of apoptosis and the ratio of *Bcl-2*/*Bax* are somehow linked with the mitochondrial permeability transition pores, which is indispensable for involved genes' release and therefore affects mitochondrial pathway apoptosis process [35]. In addition, *Caspase-3* expression was elevated to be in agreement with previous studies [36, 37]. No significant difference was found on *Smac.* This proapoptotic gene was reported to induce apoptosis through binding to inhibitors of apoptosis, relieving inhibitory effects and activating on *Caspase-3* activity [29, 38]. This may indicate that the spleen cells under Mo and Cd stress tend to resist apoptosis caused by dietary Mo and Cd. In total, changes in *Bcl-2* and *Caspase-3* suggested that the damage could involve the mitochondrial pathway in vivo.

Besides, splenic ultrastructural observation suggested that different levels of Mo and Cd induced splenic morphological changes by means of apoptosis justified by nucleus deformation, vacuolation in cytoplasma and mitochondria, as well as increasing lysosomes in splenocyte. Because of the mitochondrial damage, a series of proteins were released into the cytoplasm accelerating apoptosis execution [29]. The histological results from the experimental group showed spleen lesions with cell hemorrhage, thickening central arteries, and enhanced capsule thickness. These pathological changes verified the results above showing that Mo may present a synergistic effect on Cd in terms of spleen toxicity.

## Conclusion

In conclusion, the study revealed that the combination of Mo and Cd could aggravate spleen cell apoptosis through the mitochondrial pathway and induce pathological lesions whose mechanism is somehow linked with Mo, Cd, and Cu deposition in spleen.

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**Compliance with Ethical Standards**

**Conflict of Interest Statement** The authors declare that there are no conflicts of interest.

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