

Antitumor Effects of Cadmium Against Diethylnitrosamine-induced Liver Tumors in Mice

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Abstract

Background: Cadmium (Cd) is reported to have antitumor effects against chemical-induced liver tumors. Antitumor effects of Cd are not completely understood, but may be related to metallothionein-deficiency in tumors, which makes tumor vulnerable to necrotic effects of Cd.

Methods: Eight-week old male C57BL/6 mice were given injections of diethylnitrosamine (DEN, 90 mg/kg, and 50 mg/kg two weeks later), followed by promotion with CCl₄. CdCl₂ was administered in the drinking water (500 ppm) from 21-40 weeks after DEN initiation. Body weights were recorded and liver tumor formation was monitored via ultrasound. At the end of experiments, livers were removed, weighed, and the tumor incidence, tumor numbers and tumor size scores were recorded. Liver histology and metallothionein immunostaining were performed.

Results: After DEN injection, animal body weight decreased, and slowly recovered afterwards. Cd treatment did not affect animal body weight gain. Ultrasounds detected liver tumors 35 weeks after DEN injection. Animals were necropsied at 40 weeks. Liver/body weight ratios increased in DEN and DEN+Cd groups. Cd treatment decreased tumor incidence (71% vs 17%), tumor numbers (15 vs 2), and tumor scores (22 vs 3). Histopathology showed hepatocyte degeneration in all groups, and immunohistochemistry showed metallothionein-deficiency in liver tumors, while metallothionein stain was intensified in tumor surrounding tissues. RT-qPCR showed increases of alpha-fetoprotein in DEN-treated livers, and increases of metallothionein-2 and TNF α in Cd-treated livers.

Conclusion: Cd is effective in suppression of DEN-induced liver tumors, and the mechanisms may be related to metallothionein-deficiency in tumors and induction of TNF α to kill tumor cells.

Highlights

- HCC model was produced by diethylnitrosamine initiation and CCl₄ promotion in mice.
- Cadmium chloride in drinking water (500 ppm) from 21–40 weeks had antitumor effect.
- Cadmium decreased DEN-induced tumor incidence, tumor number, and tumor scores.
- Liver tumors are deficient in metallothionein compared to tumor surrounding tissues.
- Cadmium suppressed α -fetoprotein, increased metallothionein and TNF α in the liver.

Introduction

Hepatocellular carcinoma (HCC) is a common and lethal malignancy. Due to late diagnosis and advanced underlying liver cirrhosis, only limited treatment options with marginal clinical benefits have been available for the patients^{1,2}. Metallotherapeutics, including platinum and other metal-containing antitumor drugs are a therapeutic strategy against HCC³.

Cadmium (Cd) is a toxic heavy metal implicated in carcinogenesis ⁴, and the development of HCC ⁵. However, Cd is also effective against HCC in experimental animals ⁶⁻⁸ and in cultured liver tumor SMMC cells and their xenograft ^{9,10}. Cd-coordinated supramolecules show the potential of antitumor effects against chemoresistant malignant cells, such as Cd-pyrrhione ¹¹, CdTe/CdS ^{12,13}, TC4ATS-Cd ¹⁴, Cd-thiocarbodiazone complex ¹⁵, Cd in combination with hSmac ¹⁶, and a novel binuclear hydrazone-based Cd(II) complex ¹⁷.

HCC is often refractory to chemotherapy and radiotherapy at late stages, and is often associated with the loss of metallothionein, probably due to hypermethylation of the metallothionein genes ¹⁸⁻²⁰, and deficiency in metallothionein could make HCC vulnerable to necrotic effects of Cd, while surrounding normal tissues could be protected through MT induction ⁶. Metallothioneins are small, cysteine-rich cadmium-binding proteins that protect against cadmium toxicity ²¹.

The present study was initiated to examine the effects of cadmium via the drinking water against HCC formation initiated by diethylnitrosamine (DEN) and promoted by carbon tetrachloride. The small animal ultrasound was employed to monitor tumor formation, immunohistochemistry was used to stain MT, and the expression of the HCC biomarker α -fetoprotein, metallothionein, and tumor necrosis factor- α in the liver was determined via qPCR.

Materials And Methods

Reagents

Cadmium chloride (CdCl₂), diethylnitrosamine (DEN), and carbon tetrachloride (CCl₄) were obtained from Sigma Chemical Co (St. Louis, MO). Other reagents were of analytical grade.

Animals

Male C57BL/6 mice (6 week old) were purchased from the Animal Center Institute of Surgery Research of the Third Military Medical University (Chongqing, China). Animals were maintained in the special pathogen-free (SPF)-grade facilities at Zunyi Medical University, with controlled environment (22 ± 1°C, 50 ± 2% humidity and a 12 h: 12 h light: dark cycle) and free access to purified water and standard laboratory chow. Efforts were made to ameliorate distress and harm to animals by daily monitoring and humane treatment of animals. All animal were adequately cared for, and experimental protocols were in compliance with the Animal Management Guidelines of the Chinese Ministry of Health and approved by Animal Use and Care Committee of Zunyi Medical University (2015-01). The study is reported in accordance with ARRIVE guidelines (<https://arriveguidelines.org>).

Experimental desin

After 2 weeks of acclimation, mice (8-weeks of age) were given the first injection of diethylnitrosamine (DEN, 90 mg/kg, ip). Approximately 80% of mice survived the first DEN injection, and two weeks later, the surviving mice were given the second injection of DEN (50 mg/kg, ip), according to the protocol ²². Four weeks after initial DEN challenge (12 weeks of age), mice were given 20% CCl₄, 5 mL/kg, po, twice a week in an attempt to promote liver tumors for 4 months. Twenty-one weeks after initial DEN initiation, mice were randomly divided into DEN and DEN + Cd group. Cadmium was given via the drinking water (500 ppm) as CdCl₂ from 21-40 weeks according to the literature ^{7,8}. The body weights were monitored weekly, and ultrasound examination of liver tumor formation was performed at the 35-39th weeks after DEN initiation. At the end of 40-week experiment, mice were anesthetized with sodium pentobarbital (65 mg/kg, ip), and livers were harvested. Liver weights and tumor outcomes were recorded as illustrated below.

The visible tumors were counted and recorded. The tumor incidence (number of tumor-bearing mice), tumor numbers (total tumors found), and tumor size scores (1= tumor size < 1 mm, 2 = tumor size 1-2 mm, 3= tumor size > 2 mm) were recorded.

Ultrasound detection

The liver tumor formation was measured using B-mode ultrasound (Vevo® 2100, Fujifilm VisualSonics, Canada) with 30 MHz peak frequency linear array transducers (MS400, Fujifilm VisualSonics, mean beam frequency range of 22–55 MHz) in a digitized scale ²³.

Histopathology

Paraffin-embedded tumor and tumor surrounding tissues were cut into 3.5 µm sections. Hematoxylin-eosin (H&E) staining was used for morphometric measurement. The digitized images of slices were observed via the OLYMPUS image analysis system (OLYMPUS, Japan) at 10× and 40× magnification ²⁴.

Immunohistochemical analysis

After removing endogenous peroxidase with 3% hydrogen peroxide, sections were subjected to citric acid antigen retrieval. Sections were blocked with goat serum or rabbit serum, and then incubated with metallothionein-1 (MT-1, 1:100) primary antibody (Abcam, Cambridge, MA) overnight at 4 °C, followed by the instructions of SABC Detection System. Finally, sections were stained with DAB and counterstained with hematoxylin. The digitized images of slices were observed via the LEICA image analysis system at 40× magnification. The expression of proteins was quantitatively measured by Image ProPlus 6.0 to get the positive staining-integral optical density/area (IOD/area, density mean). 4 discontinuous areas were used to analyze the expression of proteins in blinded fashion ²⁴.

Quantitative real-time RT-PCR

Total RNA was extracted with Trizol (Takara, Dalian, China) and reverse transcribed to cDNA using PrimeScript™ RT reagent kit (Takara, Dalian, China). RT-qPCR was performed utilizing iQ™ SYBR Green Supermix (Bio-Rad, USA). The PCR cycling conditions were 94 °C for 3 min and 40 cycles of (94 °C for 15 s, 60 °C for 20 s and 72 °C for 40 s). Data were normalized to β -actin and expressed by the comparative Ct method²³. Primers for amplifying mouse genes were shown in Table 1.

Table 1

Primer sequence for RT-qPCR

Name	Access	Forward	Reverse
AFP	NM_007423	AGCAGGACTGCTCGAAACAT	AGCGAAATGTAGCAGGAGGA
β -actin	NM_007393	GATCTGGCACCACACCTTCT	GGGGTGTGGAAGGTCTCAAA
MT-2	NM_008630	CCGATCTCTCGTCGATCTTC	AGGAGCAGCAGCTTTTCTTG
TNF- α	NM_013693	TAGCCAGGAGGGAGAACAGA	TTTTCTGGAGGGAGATGTGG

Statistical analysis

Data were presented as mean \pm SE and analyzed by one-way ANOVA, followed by Duncan's multiple comparison test with SigmaPlot version 14 (San Jose, CA). $p < 0.05$ was considered statistically significant.

Results

Animal general health condition and body weight

After the initial diethylnitrosamine (DEN, 90 mg/kg, ip) administration, 80% of mice survived within 7 days, with moderate body weight reduction. After the second DEN injection, 95% of mice survived, with slight body weight reduction. Animal body weights were slowly recovered afterwards. Carbon tetrachloride promotion (20% CCl₄ 5 mL/kg, po, twice/week) was started at the 4th week after DEN injection for 4 months. Cd was given from 21-40 weeks after DEN initiation through the drinking water (500 ppm) for 19 weeks. All mice survived Cd treatment. Ultrasound was randomly performed with a few mice at the 36th week after DEN injection (15 weeks after Cd treatment) for 4 times till the 40th week of DEN initiation (19 weeks after Cd treatment). The animal body weights after Cd intervention are shown in Figure 1.

Ultrasound detection of liver tumor formation

Small animal ultrasound was used to monitor tumor formation 30 weeks after DEN injection, but no tumors were detected. After 36 weeks of DEN injection, about 40% incidence of liver tumors was detected with small sizes. At the 40th week of DEN initiation, 65% tumor incidence was detected. Figure 2 shows representative ultrasound images of DEN-induced liver tumors at the 40th weeks of DEN initiation in Control, DEN, and DEN+Cd treated mice. The ultrasound image of control mice was smooth, and in DEN-treated mice, the density was increased (arrows), indicative of liver tumors and in DEN+Cd treated mice, only one mouse showed a detectable tumor with decreased density.

Animal liver/body weight ratio

At the end of experiment, mice were euthanized and livers were collected, weighed, and liver/body ratios were calculated. Figure 3 shows the liver/body weight ratios in all groups. DEN increased the liver/body weight ratio (mg/g) from 48.5 to 53.4, while DEN + Cd further increased the liver/body weight ratio to 55.9.

Liver tumor outcome

The representative gross liver photos are shown in Figure 4, arrow indicates liver tumor in DEN-treated mice, and no tumors in Control mice and a few tumors in DEN+ Cd treated mice are evident.

The tumor outcomes are listed in Table 2 in a blinded observation. 10 of 14 DEN-treated mice had tumors with tumor incidence of 71%, and only 2 of 12 DEN+Cd treated mice had tumors with tumor incidence of 17%; there were total 15 tumors were found in DEN-treated mice, but only 2 tumors in DEN+Cd treated mice. Some tumors in DEN treated mice were large, and the tumor score in DEN treated mice was 22, while in DEN+Cd treated mice was 3. All the differences were statistically significant via Qi-Square method ($p < 0.05$).

Table 2

Tumor outcomes of DEN and DEN + Cd groups

	n	Tumor incidence	Tumor number	Tumore score
Control	5	0	0	0
DEN	14	10	15	22
DEN+Cd	12	2*	2*	3*

*Significantly different from DEN group, $p < 0.05$

Histology and immunohistochemistry

Figure 5 shows the representative histology and immunochemistry images. The top panel showed the low power (4x) HE photos, and the middle panels were enlarged (10x) photos. Liver tumors can be seen in DEN-treated mice, and hepatocellular degeneration was evident in both DEN and DEN+Cd treated mice. The bottom panel showed metallothionein immunohistochemistry. In DEN treated livers, metallothionein stain was not found in liver tumors, but strongly in tumor surrounding tissues. DEN + Cd increased the intensity of metallothionein stain. Arrows indicate liver tumors, and arrowheads indicate metallothionein stain.

RT-qPCR analysis of liver gene expression

Figure 6 shows the expression of liver genes in Control (n=5), DEN (n=14), and DEN + Cd (n=12) treated mice setting Controls as 100%. DEN treatment increase the expression of α -fetoprotein (AFP) over 2-fold (242% of Control), however, due huge individual variance, the difference was not statistically significant, while DEN+Cd had 112% of Control. The expression of metallothionein 2 gene (MT-2) was slightly decreased by DEN (73% of Control), but significantly increased by DEN+Cd by 3-fold (294% of Control). The expression of tumor necrosis factor α (TNF α) was slightly increased by DEN (142% of Control), but significantly increased by DEN+Cd (224% of Control).

Discussion

The present study demonstrated the antitumor effects of cadmium against DEN-initiated and CCl₄ promoted HCC, as evidenced by ultrasound image, tumor incidence, tumor number, and tumor score. Immunohistochemistry showed MT was deficient in HCC, but was induced in tumor surrounding tissues. RT-qPCR revealed that the HCC biomarker AFP was increased in HCC, but attenuated by Cd. Cd treatment also induced MT and TNF α in the liver. However, histology showed hepatocyte degeneration in the liver in both DEN and DEN + Cd groups.

Cadmium has been shown to exert antitumor effects against diethylnitrosamine-induced HCC in B6C3F1 mice⁶⁻⁸, including the late stage of HCC by inducing tumor necrosis⁶. The present study replicated the previous findings in C57/BL mice. Cd exposure is known to produce tumors in the lung, liver, prostate, pancreas, and injection sites⁴, and long-term Cd exposure is implicated in HCC development⁵. Paradoxically, in an effort to promote DEN-initiated HCC, Cd unexpectedly produced antitumor effects⁷. This phenomenon is common for metallo-chemotherapy agents. For example, arsenic is well-known human carcinogen, but it is also effective against hematological malignancies and certain solid tumors with arsenic-metal complexes²⁵. The use of a toxic metal/metalloid to treat chemo-resistant malignancies could be a strategy in metallo-chemotherapy.

Noninvasive imaging of HCC growth in mice with ultrasound technology²⁶ would help monitor HCC growth, the treatment efficacy and treatment duration. We have successfully applied the ultrasound in cardiovascular studies²³, and in the present study, the HCC formation could be detected 4 month after

DEN initiation (6 months of age), and at 8 months of age, 50% tumor incidence was detected, at 9 months of age, 65% tumor incidence was detected, consistent with necropsy findings. Tumor promotion with CCl₄ in the current study resulted in higher tumor incidence as compared to previous study using Cd alone^{7,8}. With the aid of ultrasound monitoring, HCC-bearing mice were killed at 40 weeks of age to examine Cd antitumor effects with reduced tumor incidence (71% vs 17%), reduced tumor numbers (15 vs 2), and tumor size scores (22 vs 3). Ultrasound dynamically helped us to define Cd treatment regimen for tumor growth inhibition.

Metallothioneins are small, cysteine-rich cadmium-binding proteins that protect against cadmium toxicity encoded by MT isoform genes^{21,27}. In human HCC, MT-1G was hypermethylated leading to reduced expression¹⁹, MT-1X and MT-2A tended to decrease with the progression of HCC²⁰, and at the late stage of HCC, MT-1A, MT-2A and MTF-1 were decreased²⁸. In present study, immunostaining for metallothionein in HCC cannot be detected, while HCC surround tissues had intense stain for metallothionein. This molecular event could be turned into an advantage for Cd to selectively kill HCC, while saving normal cells that will be protected from Cd cytotoxicity by the metallothionein induction.

Alpha-fetoprotein is a well-known biomarker of HCC, which is increased in DEN-treated mouse livers. The expression of AFP in Cd groups was attenuated; consistent with antitumor effects of Cd. On the other hand, the expression of MT-2 and TNF α in Cd-treated groups was higher compared to the model group. Cd is a potent inducer of MT gene, and both MT-1 and MT-2 genes are coordinately expressed in the mouse liver²¹, and increased MT-2 gene could protect against Cd toxicity. Induction of TNF α by Cd plays a dual role in killing tumor cells, as well as in producing liver damage²⁹.

It should be noted that antitumor effects of Cd should be carefully balanced between benefits and risks. Necrotic effects of Cd on HCC are a desired therapeutic outcome, but Cd-induced liver injury is an undesired toxic effects. In the present study, antitumor effects of Cd are accompanied by liver damage to various degrees (Figs. 4 and 5). Efforts should be made to use metals like Cd to kill malignancies while the host is tolerant to the treatment.

Collectively, this study demonstrated the antitumor effects of Cd against DEN-induced HCC in C57/B6 mice, the mechanism of action appear to be related to MT-deficiency in HCC, while normal surrounding tissues can be protected by MT. Thus, to target MT-deficiency in HCC could be a potential therapeutic strategy.

Declarations

Funding acknowledgement:

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science and Technology Department (QKHPTRC [2020]5007), the Science & Technology Plan of Zunyi (ZSKRCPT[2020]7).

Author contributions:

Yun Liu, Jie Liu and Yu Nie conceived the experiment. Yu Nie performed animal studies, including drug administration, body weight recording, necropsy, and qPCR and histology. Bo Huang and Yun-Yan Xu performed small animal ultrasound image, An-Ling Hu performed pathology and immunohistochemistry; and Yan Zou performed statistical analysis.

Consent for publication:

All authors have read and approved the final version of the manuscript for publication.

Conflict of interest/Competing interests:

We do not have conflict of interest and have nothing to declare.

Ethics approval:

All animal care and experimental protocols are complied with the Animal Management Guidelines of the Chinese Ministry of Health and approved by Animal Use and Care Committee of Zunyi Medical University (2015-01).

Availability of data and meterail:

All the data are available upon request.

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Figures

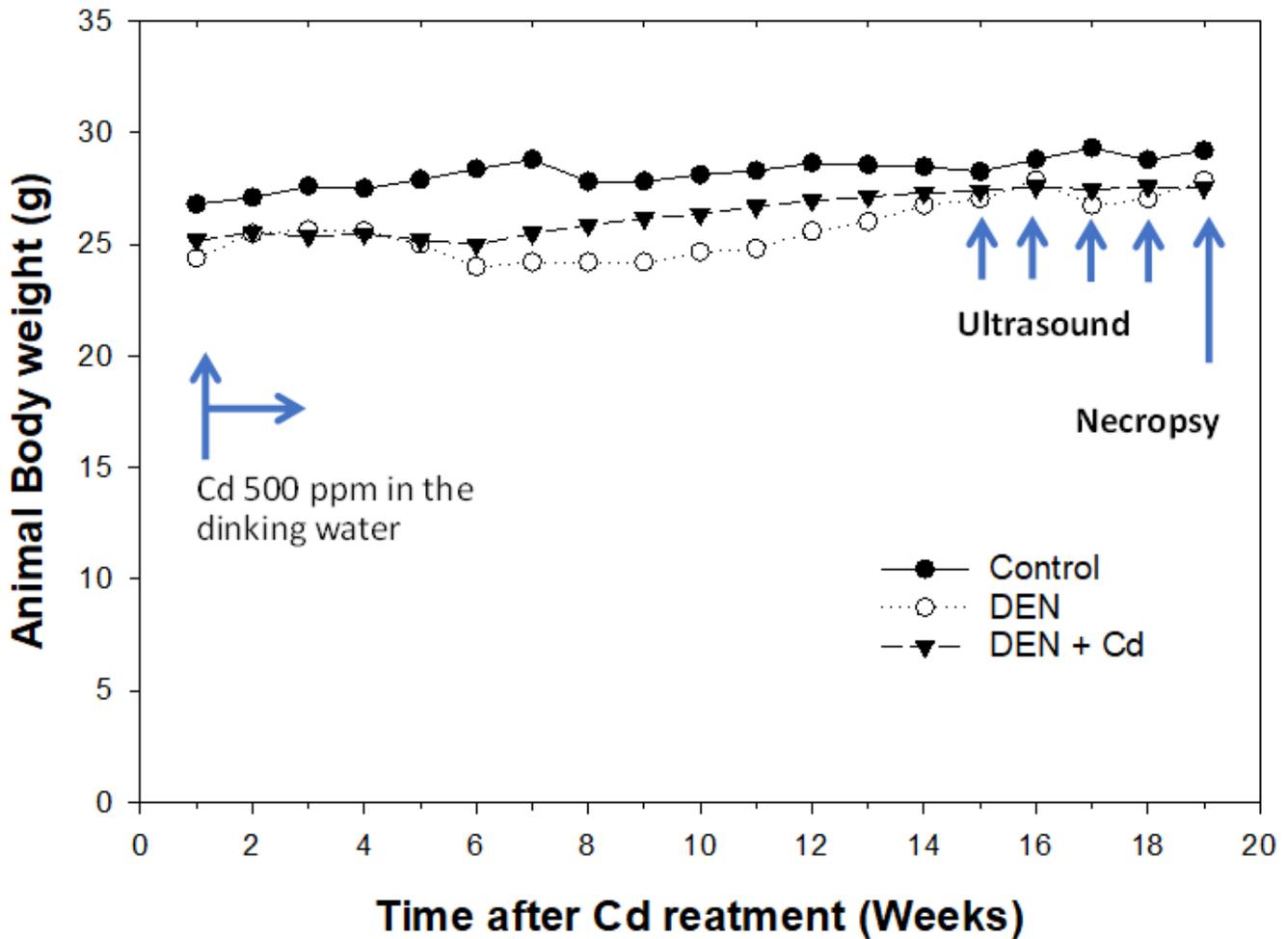


Figure 1

Animal body weights and Cd treatment procedures. Twenty-one week after dimethylnitrosamine (DEN) initiation, mice were given cadmium (500 ppm in the drinking water) for 19 weeks (40-week of DEN initiation). Data are mean of Control (n=5), DEN (n=14), and DEN + Cd (n=12).

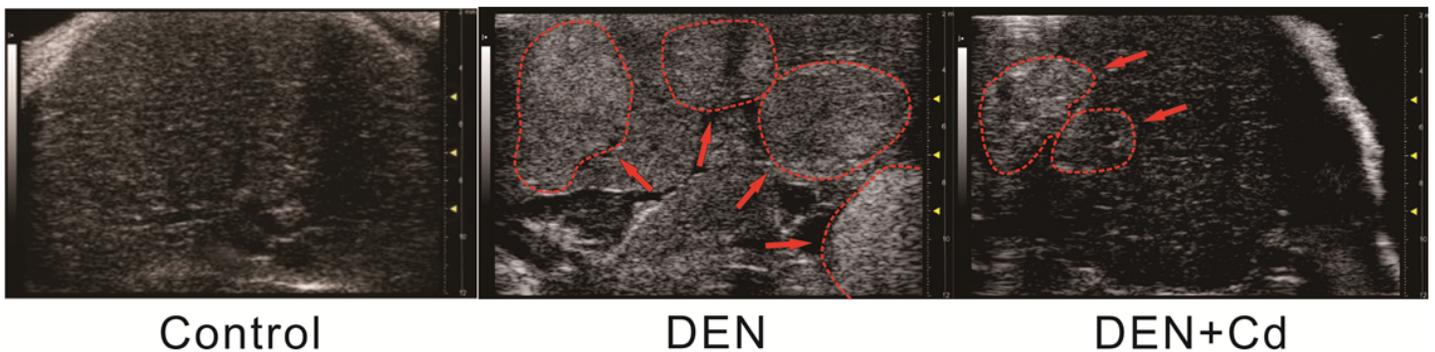


Figure 2

Represent ultrasound images of liver tumors. Mice were anesthetized, and after de-hair and the liver tumor images were performed. Arrows indicate the sense area indicating the formation of liver tumors.

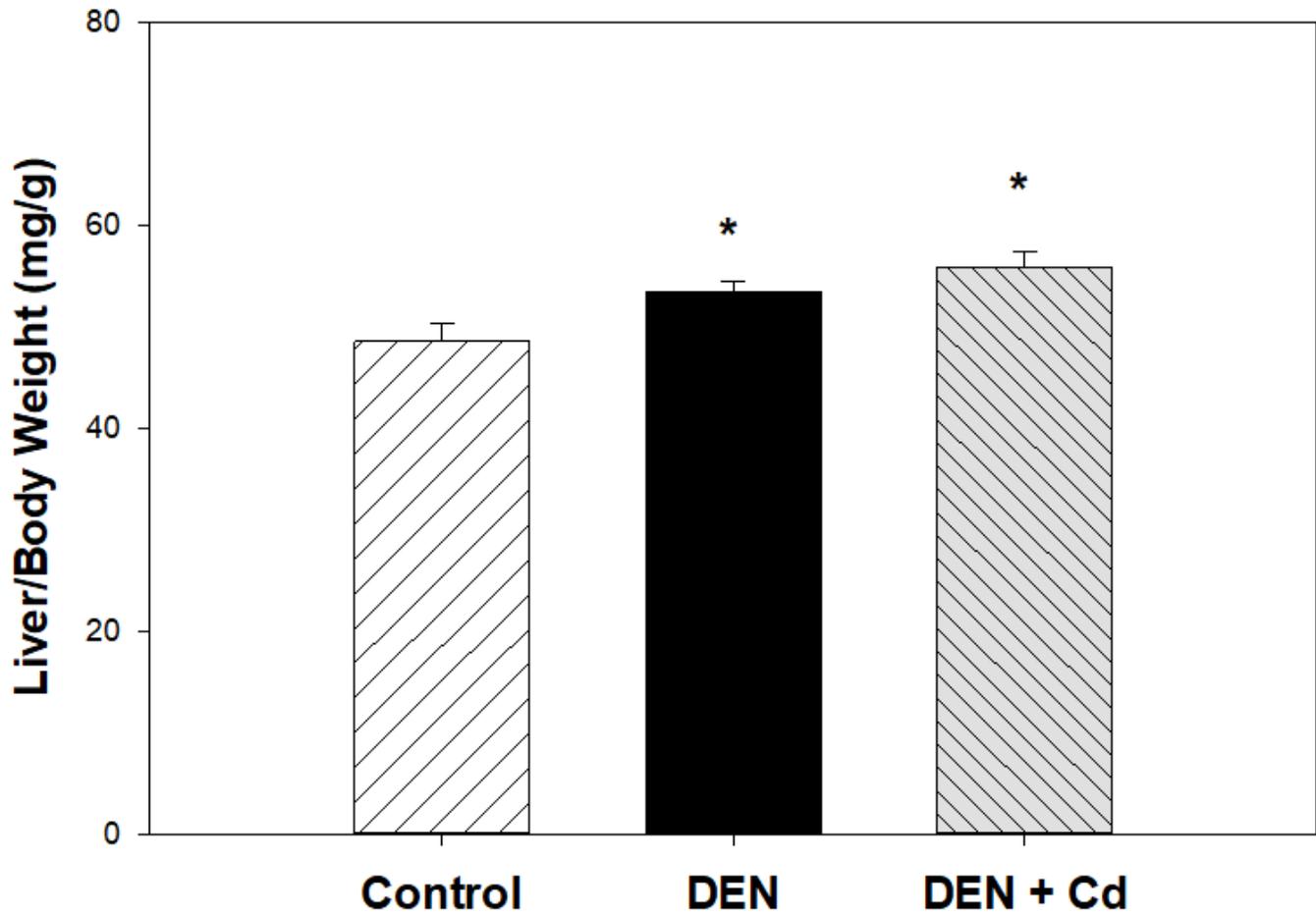
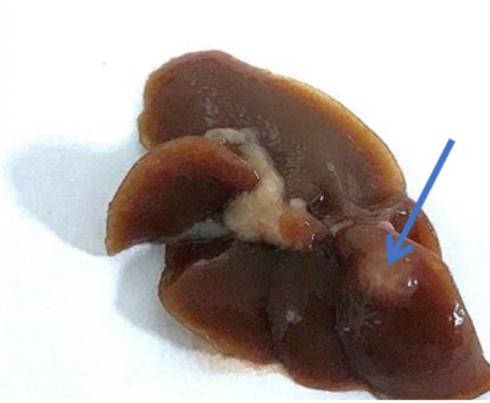


Figure 3

Animal liver/body weight ratios at necropsy. Sixteen weeks after DEN injection, mice were euthanized, and livers were collected, weighed, and liver/body weight ratios of Control (n = 5), DEN (n = 14), and Cd (n = 10) were shown. Data are mean \pm SE. *Significantly different from Controls, $p < 0.05$.



Control



DEN



DEN+Cd

Figure 4

Representative gross liver photos at necropsy. Arrow indicates liver tumor in the DEN group.

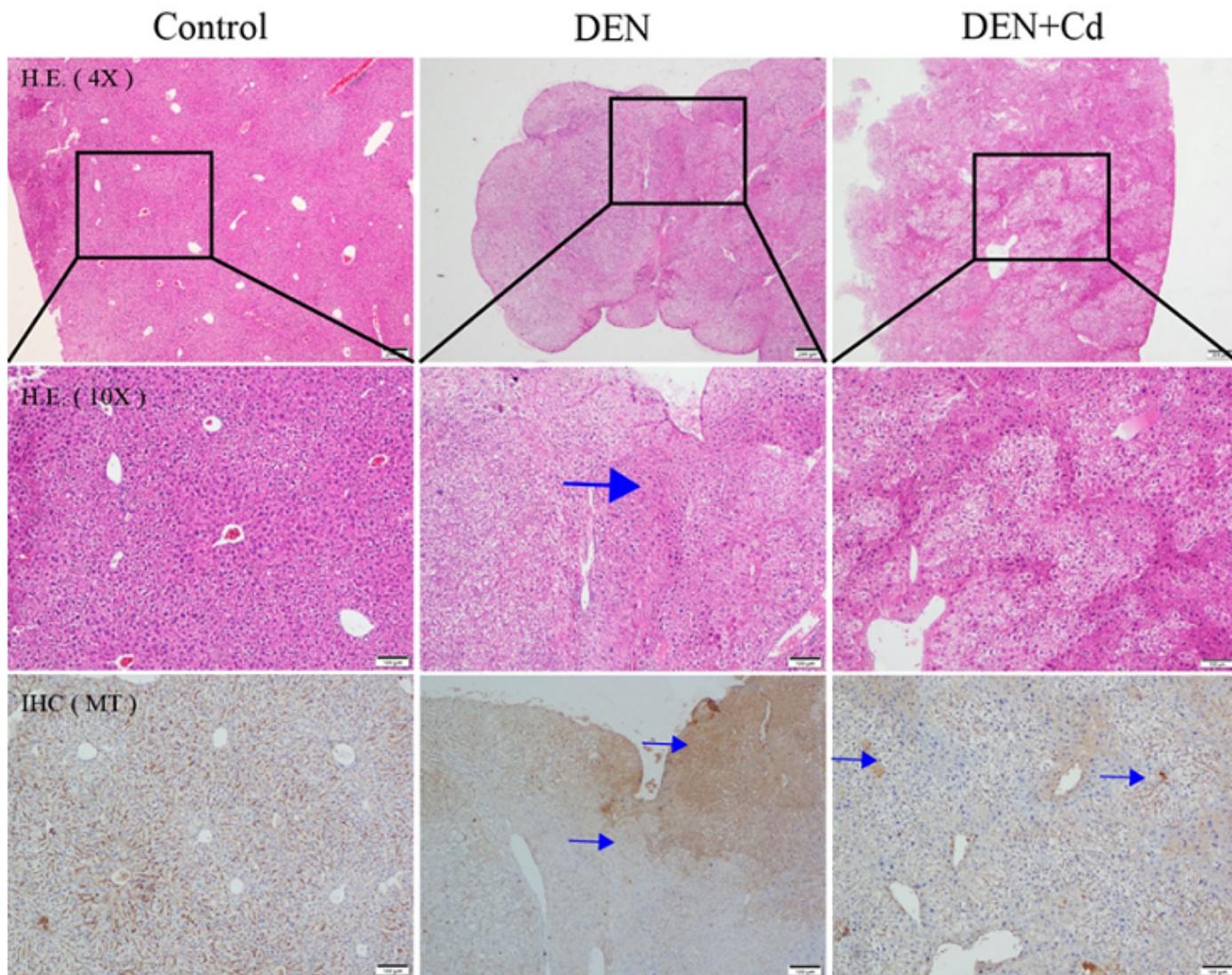


Figure 5

Representative HE and metallothionein immunocytochemistry photos. The top panel showed the low power (4x) HE photos, the middle panels were enlarged (10x) photos. The bottom panes showed metallothionein immunostain (10x) photos. Arrows in HE photos indicate liver tumors, and arrowheads in immunostain photos indicate metallothionein stain.

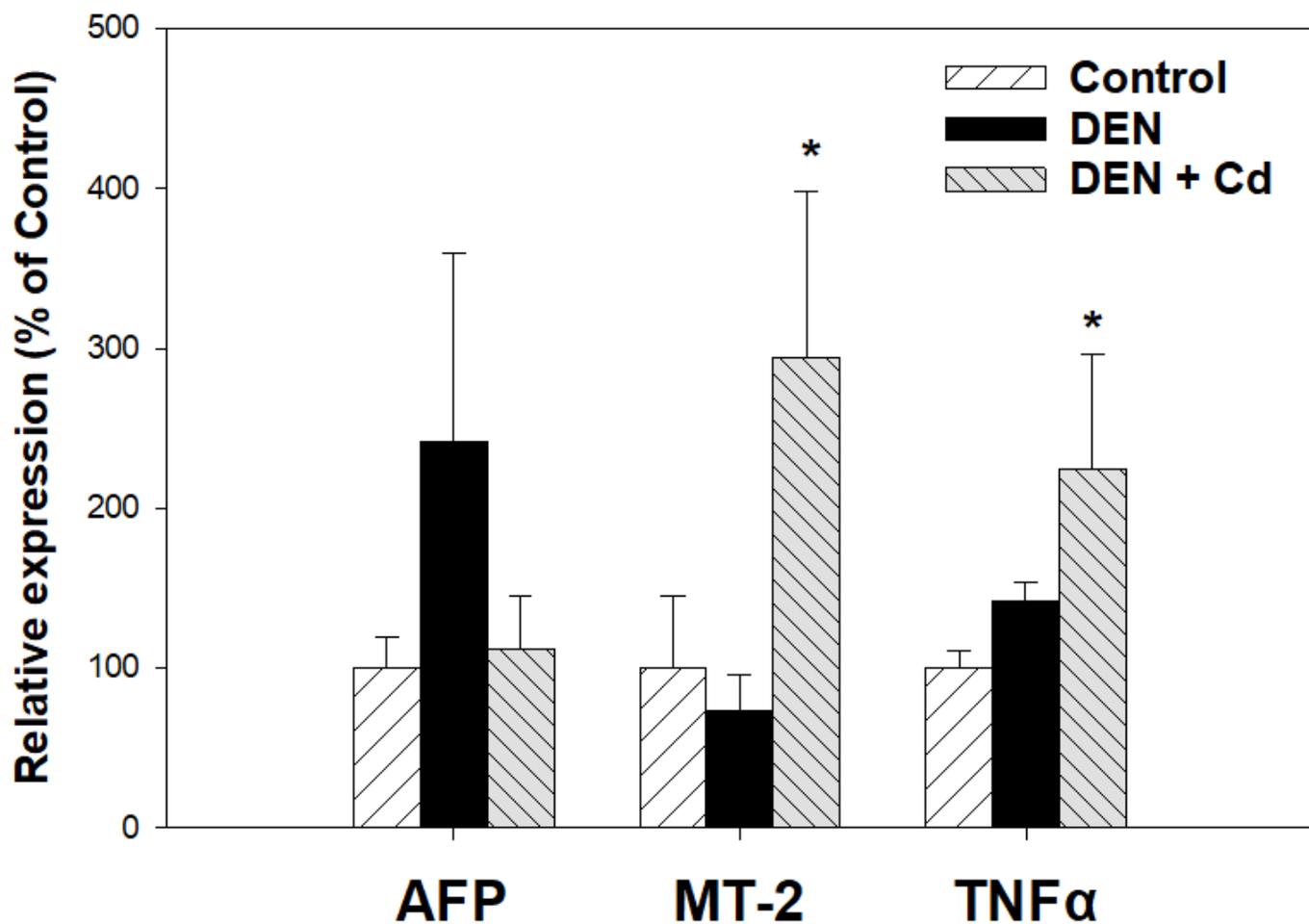


Figure 6

The expression of alpha-fetoprotein, MT-2, and TNF α via Real-time qPCR. Nineteen weeks after cadmium (1000 ppm in the drinking water) treatment, hepatic total RNA was extracted and subjected to real-time qPCR analysis. Data are mean of Control (n=5), DEN (n=14), DEN + Cd (n=12). *Significantly different from control, $p < 0.05$.

Supplementary Files

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