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Genetic and Histopathological Responses to Cadmium Toxicity in Rabbit's Kidney and Liver: Protection by Ginger (*Zingiber officinale*)

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Abstract This study aimed to examine the protective effects of ginger (G) on the genetic response induced by cadmium (Cd) and immunohistochemical expression of *Caspase3* and *MKI67* in the kidney and liver of rabbits. Male rabbits were divided into three groups; each group contains 10 animals: group (C) received basic diet and tap water for 12 weeks, the second group (Cd) received 20 mg/kg b.w $CdCl_2$ in water for 12 weeks, group (Cd+G) was given 20 mg/kg b.w $CdCl_2$ in water and 400 mg ginger/kg b.w in food for 12 weeks. Cd administration increased the activity of mRNA expression of the examined apoptotic (*Caspase3*), proliferation (*MKI67*), proto-oncogene (*C-fos*), and antioxi-dant (*GST*), while decreased the expression of anti-apoptotic (*Bcl2*). Ginger counteracted the effects of Cd in (Cd+G) group and downregulated the previously upregulated genes under Cd administration appeared in (Cd) group. The immu-nohistochemical expression of *Caspase3* and *MKI67* in the liver and kidney cells of the (C) group was shown very faint to negative reactions, strong staining in hepatocytes and the tubular epithelium in cadmium-treated group, while slight staining in some hepatocytes and tubular epithelium in co-administration with ginger in (Cd+G) group. In conclusion, ginger administration showed a protective effect against cad-mium toxicity.

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Introduction

Cadmium (Cd) has lots of health problems. Some heavy elements such as (zinc, lead, copper, and cadmium) are used in the manufacture of batteries, pigments, metal alloys, and coating of some cursive metals as well as an impurity in a lot of used materials [1, 2]. One of the significant sources of the Cd is the cigarettes' smoke [3]. Human exposure occurs by inhalation (cigarettes' smoke) and by ingestion of Cd-contaminated food or water [4, 5]; it has toxicity effect on mammalian organs like liver [6, 7], kidney [7, 8], and other body organs [9]. Cd accumulates primarily in the liver, where glutathione and metallothionein sequester the majority of intracellular Cd. Cd/metallothionein complex is slowly released from the liver in the blood circulation [10]. Because of its low molecular weight, Cd/metallothionein is freely filtered in the kidney and reabsorbed from the glomerular filtrate by megalin/cubilin receptors of PCT cells [11, 12].

Many studies showed that Cd has carcinogenic activity and produces tumors in multiple organs [9]. The International Agency for Research on Cancer (IARC) has classified Cd as a human carcinogen [13]. Many genes are responding to Cd, depending on its effects [14]. Some genes are expressed in early response to Cd like *c-fos*, *c-jun*, *c-myc* and others, which are grouped in the proto-oncogenes group, so these genes are classified as immediate early response genes (IEGs) [6, 15, 17, 18]. Another effect of Cd is an induction of reactive oxygen species (ROS) in mammalian cells [19]. ROS are the main cause of apoptosis that occur in the cells [20]. Also, Cd

stimulates an induction of many antioxidant molecules. Glutathione (GSH) which is the most important antioxidant molecule present in cells and plays a major role in Cd detoxification [21, 22]. Therefore, GSH is catalyzed by glutathione S-transferase (GST) and isozymes family [23]. So other enzymes are produced to protect cells against ROS like peroxidases, catalase, and superoxide dismutase (SOD) [24]. Furthermore, cell proliferation and apoptosis are two contradictive processes that occurred in the cell, since different genes are stimulated and expressed in each process. Many studies proved the correlation between Cd and cancer induction [15], while others mentioned the association between Cd exposure and apoptotic cells [25, 26]. Many genes were identified as apoptosis markers including Caspase gene family, which has cysteine proteases activity that is involved in the apoptotic pathway [27–29]. Caspase3 has been implicated as a key protease cleaving multiple cellular substrates and including components related to DNA repair and regulation and causing cell demise as well [30, 31]. *BCL-2* gene family is a group of genes that regulates cell's death [32] and cell's proliferation (*c-fos*, *c-jun*, *c-muc*, etc.) [15–18], while other genes are considered as immune toxicity markers like MKI67, interleukins, and CD44 [33]. MKI67 is a nuclear antigen (also named Ki-67) associated with cell proliferation and is presented throughout the active cell cycle but absent in resting cells [31, 34, 35]. The effects of Cd can be detected at different levels such as gene expression and immunohistochemical tests.

Antioxidants are natural defense mechanisms that have the power to scavenge the hazard effect of free radicals in mammals [36]. Many natural products have protective effects on mammalian tissues against various drugs or chemicals-induced toxicities [37, 38]. Ginger (*Zingiber officinale*) is one of these products which is used worldwide as a spice in food or as an unconventional medical treatment (alternative medicine), because of its enrichment of antioxidants, anti-inflammatory, and anti-cancer components, where different studies declared the role of ginger in detoxification of various chemicals and drug-induced stress conditions [37, 39–42].

The aim of the current study is to focus on the ginger effects against cadmium toxicity at molecular and immunohistochemical levels in two different organs (kidney and liver) of rabbits (*Oryctolagus cuniculus*).

Materials and Methods

Animal Treatments and Samples Collection

All animal procedures were approved by the Ethical Committee Office of the Scientific Dean of Taif University, Saudi Arabia. The experiment was carried out and described by Al-Ameer [43]. Two-month-old healthy male rabbits weighing 1350±250 g were used clinically; the animals were kept in a temperature-controlled room (24 °C) in individual cages and given a basic diet and tap water for 1 week, then divided into three groups, 10 animals in each group: group (C) was received basic diet and tap water for 12 weeks, the second group (Cd) received 20 mg/kg b.w $CdCl_2$ in water for 12 weeks, group (Cd+G) was given 20 mg/kg b.w $CdCl_2$ in water and 400 mg ginger/kg b.w in food for 12 weeks. At the end of the experimental period, the rabbits were sacrificed under chloroform anesthesia. Kidney and liver were collected from all groups and applied for RNA extraction and immunohistochemical processing.

RNA Extraction and Quantification

Total RNA was extracted according to Attia [44]. RNA samples were diluted to 40 μg/ml according to the spectroscopy quantification measurements.

Primers

Primers were designed according to gene sequences published in Genbank using free online software (Primer3Plus) presenting at the website http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/. All primers were manufactured by BIORON GmbH-Germany. Primer sequences and PCR product sizes are presented in Table 1.

Semi-Quantitative PCR Analysis

Two micrograms of the total RNA was reverse transcribed into cDNA using RevertAid First Strand cDNA synthesis kit (Fermentas, USA). The reactions were incubated for 60 min at 42 °C and terminated by heating at 70 °C for 5 min. One microliter of the cDNA was used for PCR which was carried out in the Perkin Elmer 9600 with an initial cycle of 10 min at 95 °C, 45 s at 54 °C, and 1 min at 72 °C, then followed by 40 cycles with conditions of denaturation at 95 °C for 1 min, annealing at 54 °C for 45 s, and extension at 72 °C for 1 min, followed by final extension step at 72 °C for 7 min. The reaction mixture is containing 1 unit of Ampli*Taq* Gold (PE Applied Biosystems), 1× buffer, 1.5 mM $MgCl_2$, 2.5 mM dNTPs, and 10 pmol of forward and reverse primers. The final volume of the reactions was adjusted to 25 μl. As a reference, an expression of 18S rRNA was detected by using specific primers (Table 1). A negative control containing RNA instead of cDNA was used to rule out genomic DNA contamination. The PCR products were confirmed by 2 % agarose gel electrophoresis. Band density

Table 1 Primer sequence and product size of tested genes

Gene	Accession number	Oligonucleotide sequence	Direction	Oligo position	Band size (bp)
18S	NR_033238	5′- AAACGGCTACCACATCCAAG-3′	(F)	437	485
		5′- CCCTCTTAATCATGGCCTCA-3′	(R)	921	
Caspase3	NM_001082117	5′-GCCAGAAAATACCGGTTGAA-3′	(F)	551	214
		5′-GTGGCATCAAGGGAATAGGA-3′	(R)	764	
Bcl2	XM_002713628	5′-GAGGATTGTGGCCTTCTTTG-3′	(F)	415	154
		5′-ATCCCAGCCTCCATTATCCT-3′	(R)	568	
GST	M74529	5′-GTGCAGACCAGAGCCATTTT-3′	(F)	210	222
		5′-CACCTTTTCAAAGGCAGGAA-3′	(R)	431	
C-fos	AB020214	5′-AGTCCGAGGAGCCTTTCAGT-3′	(F)	236	206
		5′-CCATCTCGGCATAGAAGGAA-3′	(R)	441	
MKI67	XM_008274847	5′-GTCACCGAGAGGCAGAGAAC-3′	(F)	7621	232
		5′-TTTGCCCTTCTTCCACATTC-3′	(R)	7852	

of PCR products was measured using freeware (ImageJ 1.48v) presenting at the website http://imagej.nih.gov/ij/.

Immunohistochemical Techniques

The collected kidney and liver of all three rabbit groups were sliced and fixed in 10 % buffered formalin solution, dehydrated in ascending grades of alcohols, cleared in xylene, and embedded in paraffin. The samples were casted, then sliced into 5 μm in thickness, and placed onto glass slides. Sections were deparaffinized in xylene and rehydrated through graded concentrations of ethanol. After the blocking of endogenous peroxidase activity with 3 % hydrogen peroxide for 15 min, the sections were heated in 0.01 mol/L citrate buffer in a microwave pressure cooker for 20 min. The slides were allowed to cool down to room temperature, and nonspecific binding was blocked with normal mouse serum for 30 min. Then, the slides were incubated overnight at 4 °C with MKI67 (clone MM1, 1:100; Novocastra Laboratories Ltd, Newcastle, UK) and rabbit polyclonal antibody against cleaved Caspase3 (Asp175, 1:50; Cell Signalling Technology, Inc., Beverly, MA, USA). After several rinses in TBS, the sections were incubated with biotinylated anti-mouse IgG at a dilution of 1:200 for 45 min at room temperature, followed by peroxidase conjugated avidin-biotin complex and 3,3′-diaminobenzidine (DAKO, Carpinteria, CA, USA). The sections were then counterstained with Mayer's hematoxylin and analyzed by standard light microscopy [45, 46].

Statistical Analysis

Results are expressed as means±SEM of 10 different rabbits per each group. Statistical analysis was done using ANOVA and Fisher's post hoc test, with $p<0.05$ being considered as statistically significant.

Results

Genetic Response to Cd a ıistration of Ginger

Five genes (*Caspase3*, *Bcl2*, *GST*, *C-fos*, and *MKI67*) were used to test their expression in rabbit's kidney and liver in response to cadmium toxicity and administration of ginger (as an antioxidant material). Gene expression was measured using semi-quantitative PCR technique, and the expression of 18S was used as an internal reference.

The expression of kidney apoptotic *Caspase3* was significantly increased under Cd treatment, while the co-administration of Cd plus ginger (Cd+G) decreased the gene expression in comparison with Cd treatment (Fig. 1a), as well as in liver, as *Caspase3* was overexpressed in response to Cd treatment and to (Cd+G), respectively (Fig. 1b).

Kidney and liver anti-apoptotic *Bcl2* gene was significantly reduced in response to Cd treatment, while it was slightly reduced in (Cd+G) group in comparison with the Cd group (Fig. 1c, d).

The expression of antioxidant *GST* gene was significantly increased in kidney in response to Cd treatment other than the control. The increase in *GST* expression was more significant in the (Cd+G) group than that in the Cd group (Fig. 1e). On the other hand, the liver *GST* expression was slightly reduced in (Cd+G) group in comparison with Cd group (Fig. 1f).

The expression of kidney and liver proto-oncogene *C-fos* was significantly increased in response to Cd treatment, while it showed less expression in the (Cd+G) group (Fig. 2a, b).

The expression of the immune toxicity *MKI67* gene was also significantly increased in kidney and liver tissues in response to Cd treatment in comparison with the control, while

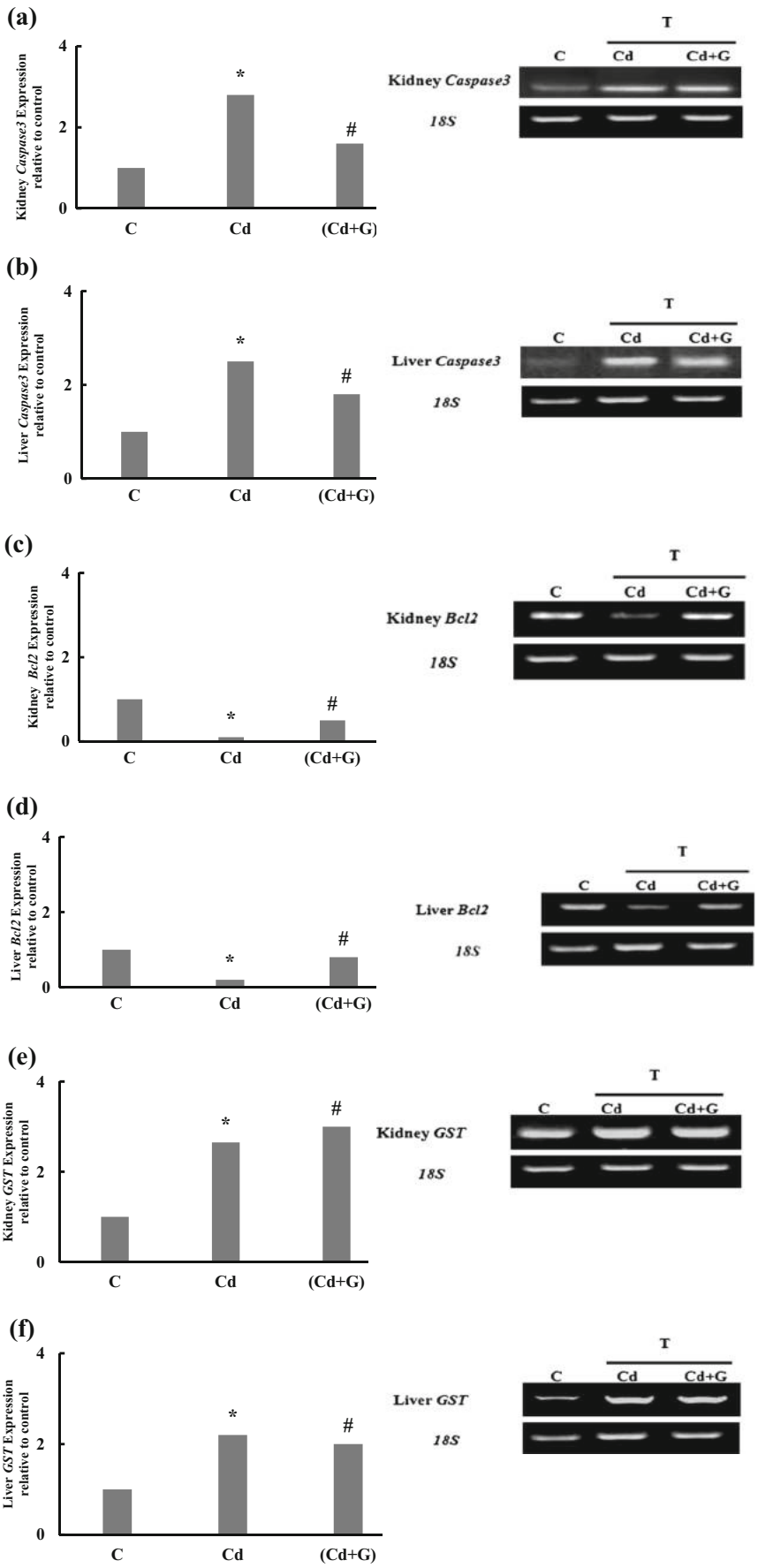

Fig. 1 Gene expression relative to control, (*C*) negative control, (*T*) treatments, (*Cd*) cadmium, and (*Cd+G*) double treatment with cadmium and ginger. Expression of *Caspase3* in kidney (**a**), in liver (**b**), expression of *BCL2* in kidney (**c**), in liver (**d**), expression of *GST* in kidney (**e**), and in liver (**f**). Values are statistically significant at $*p<0.05$ vs. control, $^{\#}p<0.05$ vs. Cd and data represent the means±SEM

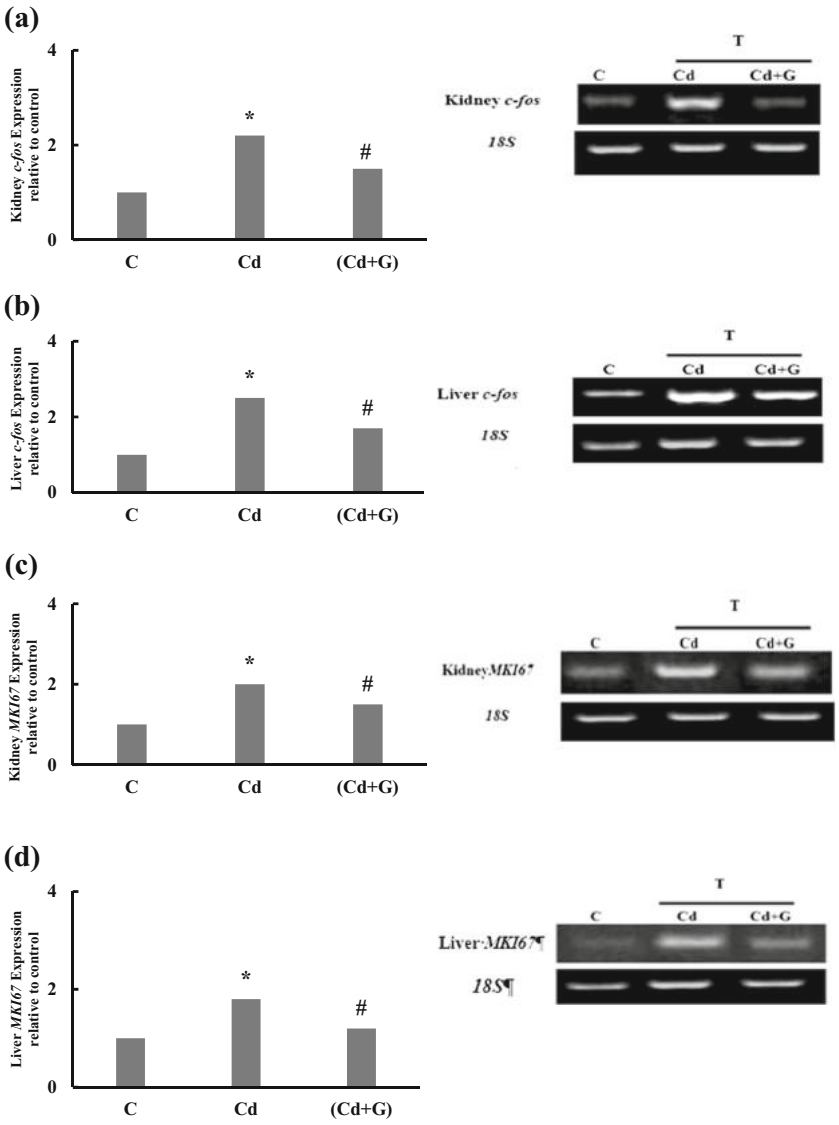

Fig. 2 Gene expression relative to control, (*C*) negative control, (*T*) treatments, (*Cd*) cadmium, and (*Cd+G*) double treatment with cadmium and ginger. Expression of *c-fos* in kidney (**a**), in liver (**b**), expression of *MKI67* in kidney (**c**), and in liver (**d**). Values are statistically significant at $*p<0.05$ vs. control, $^{\#}p<0.05$ vs. Cd and data represent the means±SEM

the increase was less in the (Cd+G) group in comparing with Cd group in both organs (Fig. 2c, d), respectively.

Immunohistochemical Changes in Liver (Caspase3)

The immunohistochemical expression of liver Caspase3 in the group (C) showed very faint to negative reactions (Fig. 3a), while the immunohistochemical expression of Caspase3 was positively strong in some sporadic hepatic cells, especially around the central vein of the Cd group (Fig. 3b). The immunohistochemical expression of Caspase3 was increased and appeared strong with positive reaction in some cells and moderate reaction in other cells (Fig. 3c). The (Cd+C) group showed slight staining while the hepatic cord cells showed normal architecture (Fig. 3d).

Immunohistochemical Changes in Kidney (Caspase3)

The immunohistochemical expression of Caspase3 in the kidney of the (C) group showed negative reaction in the cells of the renal tubules and renal corpuscles (Fig. 4a). The immunohistochemical expression of Caspase3 in the kidney of the (Cd) group showed positive reaction in the cells of the renal tubules (PCT & DCT), while faint reaction in other renal tubules, and the flat squamous cells of the Bowman's capsules showed faint reaction (Fig. 4b). Some cells of the renal tubules showed vacuolations and negative immunostaining, while the other cells showed positive immunostaining (Fig. 4c). The immunohistochemical expression of Caspase3 of the (Cd+G) group showed faint immunostaining in the tubular cells (Fig. 4d).

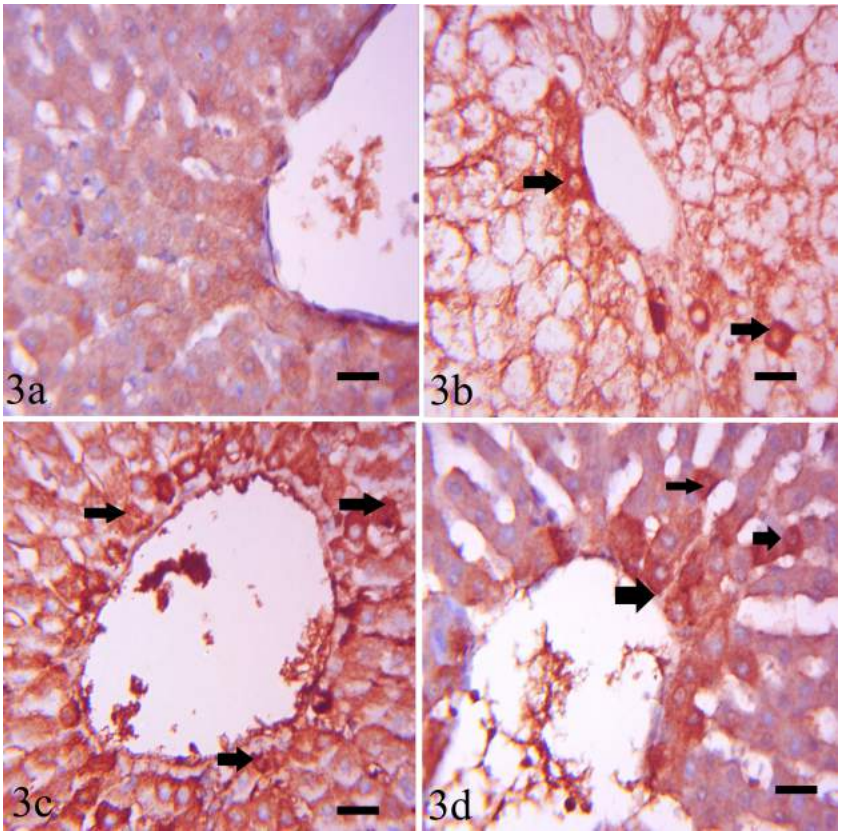

Fig. 3 Photomicrograph of the liver showing immunohistochemical expression of caspase3. **a** Control group showed negative reaction, *bar*= 10 μm; **b** Cd-treated group showed positive immunohistochemical staining in hepatocytes around central vein (*arrows*), *bar*=10 μm; **c** strong immunohistochemical staining in hepatocytes in cadmium-treated group (*arrows*), *bar*=10 μm. **d** Slight immunohistochemical staining in some hepatocytes (*arrows*) in co-administration with ginger group, *bar*=10 μm

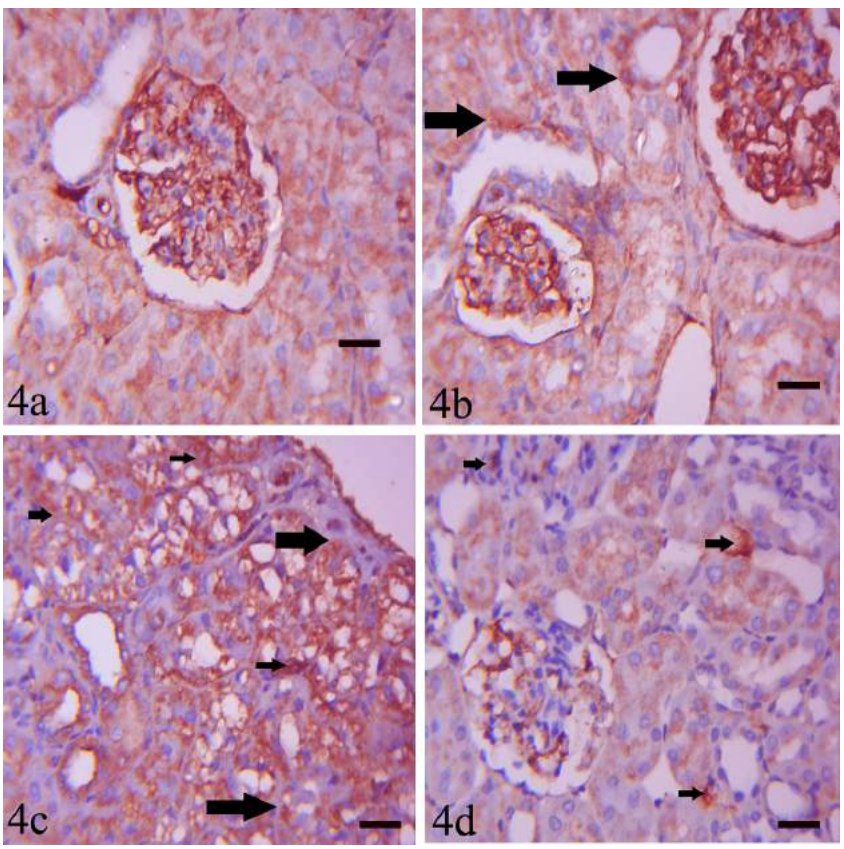

Fig. 4 Photomicrograph of the kidney showing immunohistochemical expression of caspase3. **a** Control group showed negative reaction, *bar*= 10 μm; **b** Cd-treated group showed positive immunohistochemical staining in the epithelium of renal tubules (*arrows*), bar=10 μm; **c** strong immunohistochemical staining in the tubular epithelium in cadmium-treated group (*arrows*), *bar*=10 μm. **d** Slight immunohistochemical staining in some epithelial cells (*arrows*) in co-administration with ginger group, *bar*=10 μm

Immunohistochemical Changes in Liver (MKI67)

The immunohistochemical expression of MKI67 in the liver's cells of the (C) group showed a negative reaction (Fig. 5a), while the immunohistochemical expression of MKI67 in the hepatic cells of the (Cd) group showed a positive one (Fig. 5b, c). The immunohistochemical expression of MKI67 in the (Cd+G) group showed single positive cells especially around the central vein (Fig. 5d).

Immunohistochemical Changes in Kidney (MKI67)

The immunohistochemical expression of MKI67 in the kidney of the (C) group showed a negative immunostaining reaction (Fig. 6a), while the immunohistochemical expression of MKI67 in the (Cd) group showed moderate reactions in the brush border of the cuboidal cells of the renal tubules (PCT & DCT) and faint reaction in the flat cells of the Bowman's capsule (Fig. 6b). The reaction showed a strong positive immunostaining in the cells of the collecting tubules of the nephron (Fig. 6c). The immunohistochemical expression of MKI67 in the (Cd+G) group showed very faint reaction in the single cell of the renal tubules (Fig. 6d).

Discussion

Different remarkable deleterious effects can be recognized in all mammalian organs that are exposed to cadmium toxicity. The expression level of marker genes which responds to different stresses including heavy metal toxicity, oxidative stress, and carcinogenesis can be used to detect the response to these stresses. Cadmium stimulates free radical production, resulting in oxidative deterioration of lipids, proteins, and DNA [47]. An apoptosis is a consequence of reactive oxygen species (ROS) that are produced in cells. Caspase3 is aprototypical enzyme that becomes activated during apoptosis in a wide variety of tissues [30]. Quantification of *Caspase3* expression may constitute a good method for measuring apoptotic activity in prostate cancer and its precursors [48].

Our results showed that cadmium increased the expression of *Caspase3*, while ginger reduced its expression; this result was detected by measuring the *Caspase3* expression and is also shown in the immunohistochemical test as a very faint to negative reaction seen in hepatocytes and the tubular epithelium of (C) group, while in the (Cd) group, the staining reaction was strong in the hepatocytes and the tubular epithelium cells; nevertheless, the reaction was reduced in the (Cd+G) group. These results reflect the curative effect of ginger on tissues under toxicity of Cd. Also, some studies showed that

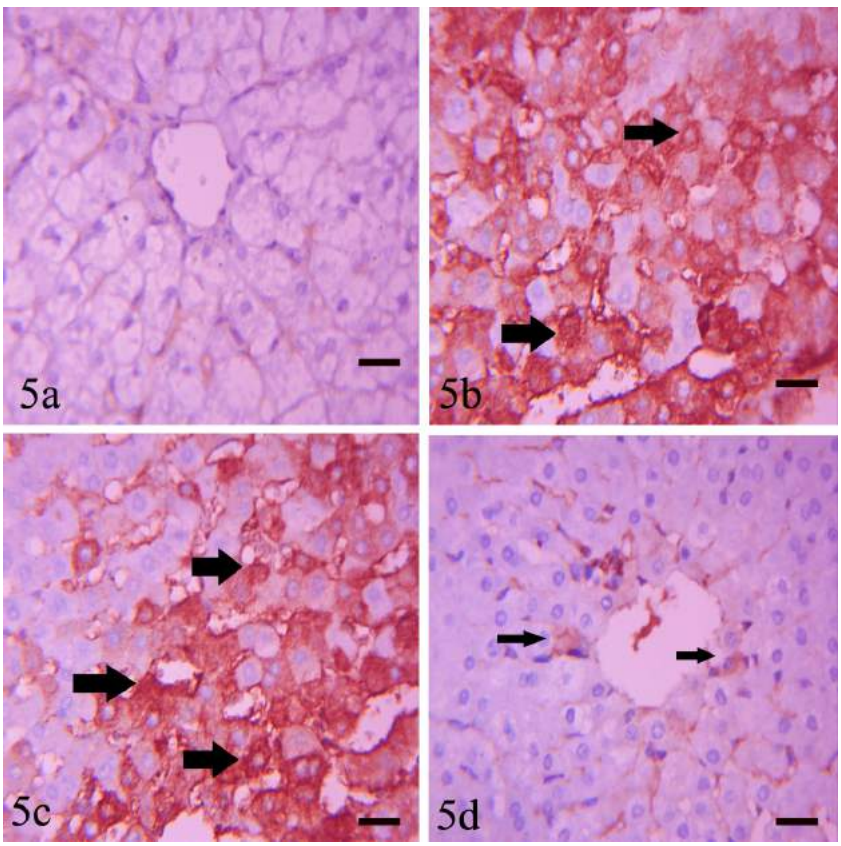

Fig. 5 Photomicrograph of the liver showing immunohistochemical expression of MKI67. **a** Control group showed negative reaction, *bar*= 10 μm; **b** Cd-treated group showed positive immunohistochemical staining in hepatocytes (*arrows*), *bar*= 10 μm; **c** strong immunohistochemical staining in hepatocytes in cadmium-treated group (*arrows*), *bar*=10 μm. **d** Slight immunohistochemical staining in some hepatocytes (*arrows*) in co-administration with ginger group, *bar*=10 μm

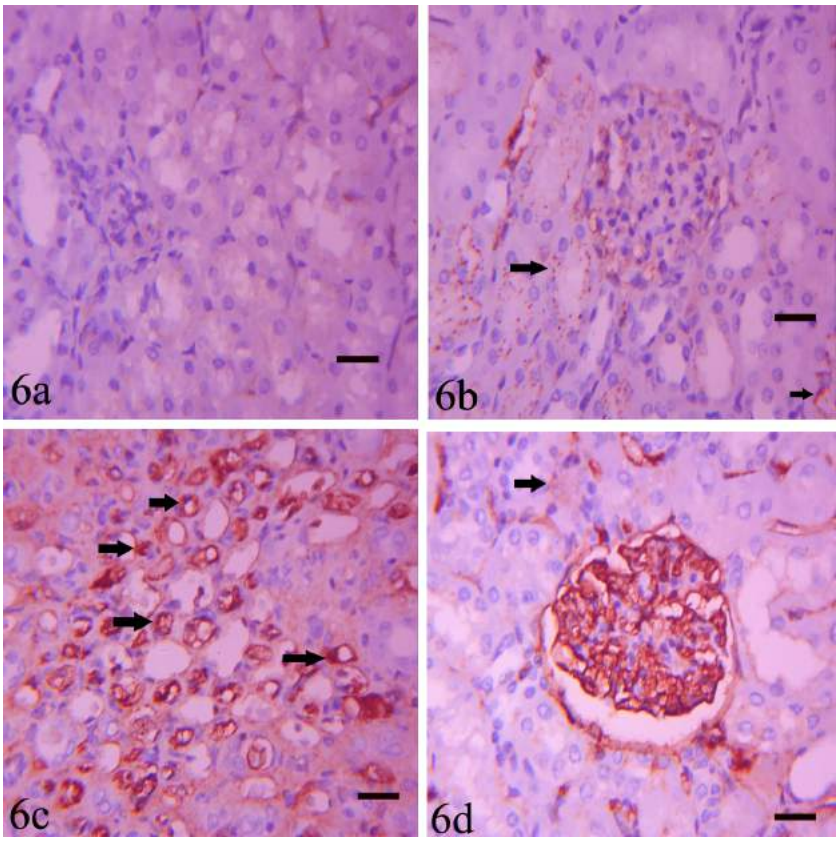

Fig. 6 Photomicrograph of the kidney showing immunohistochemical expression of MKI67. **a** Control group showed negative reaction, *bar*= 10 μm; **b** Cd-treated group showed positive immunohistochemical staining in the epithelium of renal tubules (*arrows*), *bar*=10 μm; **c** strong immunohistochemical staining in the tubular epithelium in cadmium-treated group (*arrows*), *bar*=10 μm. **d** Slight immunohistochemical staining in some epithelial cells (*arrow*) in co-administration with ginger group, *bar*=10 μm

ginger induces apoptosis depending on the ginger components. Yagihashi [49] found that 6-gingerol inhibited the proliferation of hepatoma cells in culture and thought that this may be due to cell cycle arrest and apoptosis induction. In addition, it was found that 6-gingerol induces apoptosis in human cancer cells by increasing the *p53* and *Bax* levels, Caspase activation, and increasing in apoptotic protease-activating factor-1 (Apaf-1) [50]. Meanwhile, 6-Paradol and other structurally related derivatives were found to induce apoptosis in an oral squamous carcinoma cell line through a Caspase3-dependent mechanism [51]. The possible explanation of the discrepancy between our results and the results of Yagihashi is that the cadmium stimulates the production of ROS which results in apoptosis, as ginger has antioxidant properties that reduce the deleterious effects of ROS [52]. Therefore, this explanation is reinforced by measuring the expression of the *GST* gene (done in this investigation) under the administration of ginger, which disclosed that ginger significantly upregulates the expression of the *GST* gene; this finding was congruous with the results of Min [53]. Our results declared that ginger increased the expression of *Bcl2* more than what appeared in the case of (Cd) group; this may be due to the detoxification power of ginger against Cd toxicity (Cd+G) group. The treatment with ginger alone did not show any significant results when compared with the results of (C) group (unpublished data). There are three ways of body cleaning from toxins: detoxification in liver, rid through urine and feces, and finally through sweating. Ginger helps in body cleaning by increasing body sweating and stimulates the production of the antioxidants [37].

MKI67 gene is a marker of cell proliferation and is related to the proliferation of tumor cells; therefore, its coding protein is a DNA-binding protein. *MKI67* is expressed in the nuclear matrix of cells during the late G1, S, G2, and M phases of the cell cycle; it peaks in the G2 and early M phases [35]. Cadmium stimulates the production of *MKI67* mRNA and has a carcinogenesis activity. So, previous studies have found that the enhanced expression of *MKI67* in kidney tissue was closely related to kidney cancer staging, grading, and prognosis as well [54]. The results obtained in our present study show that the treatment with Cd induces an increase of the *MKI67* and *C-fos* expression, whereas ginger inhibited the increased expression of both genes as shown in (Cd+G) group. The immunohistochemical test reflected these results as strong staining reaction for MKI67, which appeared in hepatocytes and the tubular epithelium of (Cd) group, also slight staining reaction in hepatocytes and the tubular epithelium of (Cd+G) group. We suggest that ginger reduced the expression of *MKI67* and *C-fos* by decreasing the toxicity effect of cadmium, as this suggestion was agreed with Baiomy [55] who stated that ginger and zinc chloride mixture reduces the *MKI67* expression on the liver and kidney alterations that

was induced by malathion toxicity. The results of gene expression experiment were congruous with the results of immunohistochemical experiment. In conclusion, the present study showed, besides the response of genes expression, the immunohistochemical expression of Caspase3 and MKI67 was strong in hepatocytes and the tubular epithelium in (Cd) group, while slight staining in some hepatocytes and tubular epithelium in (Cd+G) group. The possible cause of the increase may be related to cellular damage by different routes. Hence, protective effect of ginger against cadmium toxicity appeared in rabbit's kidney and liver after cadmium administration.

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