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Role of Oxidative Stress, Apoptosis and Autophagy in Cadmium-induced Renal Injury in Rats: Renoprotective Effect of Ghrelin

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- **LC3**
- **Caspase-3**

Abstract

Background: Kidney diseases are one of the most common disorders of the modern life. Cadmium (Cd)-induced kidney damage is a common cause of chronic renal failure. Several mechanisms have been postulated to explain the mechanisms underlying Cd induced renal injury such as oxidative stress, apoptosis and autophagy. The aim of the present study was to examine the effect of acylated ghrelin (AG) on renal damage induced by Cd and possible role of autophagy, oxidative stress and apoptosis **Methods:** Thirty male Sprague Dawley rats were subdivided into 3 equal groups; Group I (control) was the vehicle control, Group II (Cd) group in which rats were subjected to 5 mg Cd/Kg/d via gastric gavage for 4 weeks, Group III (AG) as group II but rats were treated with 3 nmol of ghrelin/animal per day via subcutaneously route for 4 weeks. By the end of experiment, serum levels of creatinine ,Na+ and K, the levels of lipid peroxidations marker (MDA) and antioxidants (GSH) in kidney tissues, the level of kidney injury molecule-1 (Kim-1) in urine and the expression of caspase-3 (apoptotic marker) and LC3 (autophagic markers) in kidney tissues were measured. **Results:** Cd administration caused significant increase in serum creatinine and k, urine KIM1 with significant increase in kidney tissues LC3, caspase 3 and MDA that was concomitant with a significant decrease in GSH in ($p< 0.05$). Administration of AG with Cd caused significant improvement in the studied parameters. **Conclusion:** we concluded that AG attenuates the renal injury induced by cadmium, which might be due to attenuation of apoptosis, oxidative stress and autophagic process in kidney tissues.

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INTRODUCTION

Chronic kidney disease (CKD) is a major public health problem which represents a new epidemic of chronic conditions that replaced infections and malnutrition as leading causes of mortality during the 20th century. In Egypt, CKD is increasingly recognized as a public health problem and holding the 7th cause of death (1, 2, 3). The heavy metal cadmium (Cd) is considered as the most common environmental and accidental pollutant in developing countries that may adversely affect human health. Exposure is largely via the respiratory or gastrointestinal tracts; important non-industrial sources of exposure are cigarette smoke and food (from contaminated soil and water) (4). The kidneys are well recognized as the primary organ targeted by chronic Cd exposure where 50% of the total Cd in vivo is accumulated (5). It has reported that cadmium has a nephrotoxic effect in which chronic kidney disease in the form of interstitial nephritis is the main pathologic insult that results in loss of kidney reabsorptive and secretory functions and eventually lead to renal failure and end-stage renal disease (ESRD) (5,6,7).

The mechanisms underlying the renal injury induced by Cd are complex and multifactorial that might include oxidative stress (8, 9, 10, and 11) and apoptosis (12, 13). However, the specific molecular mechanisms underlying these effects are not yet fully understood (14, 15). Unlike apoptosis, autophagy is mainly an adaptive process by the cells that is up regulated in response to environmental stressors to maintain cell survival and repair .Autophagy, begins with the formation of a double-membrane vesicle termed ‘‘autophagosome’’ that engulfs a part of cytosol (16). Which then fuses with a lysosome to yield an ‘‘autolysosome’’ which is eventually degraded for the synthesis of new molecules or organelle. Depending on the environmental stressors and cell type, autophagy pathway could act as a survival mechanism by clearing the damaged organelles and toxic protein aggregates that may otherwise induce cellular apoptosis. However, if autophagy is massive and persistent, it could deplete the cells of organelles and critical proteins and kill severely damaged cells through type II cell death which is caspase-independent (17).

Kidney injury molecule-1 (KIM-1) is a type 1 cell membrane glycoprotein composed of six cysteine immunoglobulin-like and mucin domain (18). KIM-1 is normally undetectable in normal urine but it is expressed at high levels following kidney insult (19; 20; 21). Raised KIM-1 level was reported in human renal conditions associated with renal scarring, inflammation and dysfunction (22). Moreover, it was reported that KIM-1 could be used as a biomarker for Cd as it is correlated with urinary -Cd and has the potential to be used as an early indicator of Cd toxicity (23, 24).

Ghrelin is a novel peptide of 28-amino-acid-peptide and is a natural ligand of the growth hormone secretagogue receptor (25). The two main types of ghrelin; acylated ghrelin (AG) and des-acyl ghrelin (26). Ghrelin classic functions include: food control, energy regulation, and growth hormone release. Several studies demonstrated other pleiotropic actions for ghrelin such as antioxidant actions (27, 28.), anti-apoptotic (29, 30) and autophagy modulator (30).

Ghrelin has beneficial effects against many experimental models of renal diseases such as: ischemia reperfusion (31), ischemic acute renal failure (32) and endotoxemia-induced acute kidney injury (33) .In addition, ghrelin also is reported to be beneficial in patients with chronic renal conditions (34, 35). Moreover, ghrelin mitigated Cd toxic effects on rat testis (28). However, to the best of our knowledge, no study investigated ghrelin renoprotective effect against Cd- induced renal damage and autophagy process. So, the present study was designed to investigate the effect of ghrelin on Cd – induced renal damage if any and the possible role of oxidative stress, apoptosis and autophagy in the potential renoprotective effect for ghrelin.

Materials and Methods

Thirty male albino Sprague Dawley rats weighing 180-200 g were used in the present study. The animals had free access to food and water and were housed in standard cages with a 12-hour light-dark cycle.in standard cages at Department of Physiology, Mansoura Faculty of Medicine, maintained on a 12 h light-dark cycle and fed on standard diet and water ad libitum. The animals were adapted to these conditions for at least 2 weeks before being used in the experiments. All experimental procedures were conducted in compliance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by our local Committee for Animal Experiments (code # R/17.10.97).

Study design

Rats were randomly allocated into 3 groups (each 10 rats) as follow;

Group I (control or vehicle group) in which rats received equivalent volumes of sterile 0.9 % NaCl solution by gavage.

Group II (Cd group): rats received Cd at a dose of 5 mg /kg/d by gastric gavage for 4 weeks (15).

Group III (ghrelin (AG) group): as group II, but rats were treated with acylated ghrelin at a dose of 3 nmol /animal per day via subcutaneously route for 4 weeks (37).

Collection of blood and urine samples and harvesting kidney tissues

One day before the rat sacrifice, rats were placed individually in a metabolic cage, fasted but had free access to water. For each rat, a 24 hours urine was collected in a sterile container and stored at – 20°C for biochemical analysis of KIM-1. By the end of experiment, the animals were anesthetized with phenobarbital [12 mg/ kg i.p.] and the blood collected by heart puncture. Then rats were sacrificed by cervical dislocation, the abdomen was opened quickly, and the kidney was immediately removed and washed thoroughly with ice-cold 0.9 % sodium chloride solution and weighted and divided into 2 halves; one half was stored at -20°C for assay of markers of oxidative stress and the second half was stored in formalin 10% for histopathological and immunohistochemical examination studies.

Measurement of serum creatinine, Na, and K

The collected blood samples were allowed to clot for 30 min, serum was separated by centrifugation at 2500 rpm for 15 min and stored at - 20°C for analysis of serum creatinine, Na and K level. The serum creatinine level was estimated according to the manufacturer's instructions (Bio-Diagnostic

Dokki, Giza, Egypt), while serum Na and K were measured using (Biomed Diagnostics- EGY-Chem., Egypt).

Measurement of KIM1 in urine

A 4-ml urine was centrifuged at 3000 rpm for 20 minutes at room temperature. The supernatant was stored at -20°C for downstream KIM-1 analysis using Rat Kim-1 Sandwich ELISA Kit (Catalogue # MBS355395) according to the manufacturer's instructions (Rat Kim-1 ELISA Kit, Boster immuneboster. Fremont, CA, USA)

Assay of lipid peroxidations marker (MDA) and antioxidants (GSH activity) in kidney tissues

About 50 -100 mg of kidney tissues will be homogenized in 1-2 ml cold buffer (50 mM potassium phosphate, pH 7.5,1 mM EDTA) using mortar and pestle then centrifuged at 4,000 rpm for 15 minutes at 4°C. The supernatant was kept at -20 °C until the time of analysis of oxidants and antioxidants. Malondialdehyde (MDA) and reduced glutathione (GSH) in the supernatant of kidney homogenates were measured using a colorimetric method according to the manufacturer's instructions (Bio-Diagnostics, Dokki, Giza, Egypt).

Histopathological examination of the kidney

The dissected left kidneys were cut into small pieces after its dissection out, kept in fixative (10% neutral buffered formalin) for about 1 week, washed, dehydrated with alcohol of different grades, then cleared by xylene and embedded in paraffin wax to form hard block. 5-µm thick sections from hard block were mounted on glass slides and subjected to the following staining procedures: Hematoxylin and Eosin stain for detection of any histopathological changes, Masson's Trichrome stain for evaluation of collagen and extracellular matrix (ECM) accumulation and interstitial fibrosis.

Immunohistochemical examination for LC3 and caspase-3

For immunohistochemical examination, the slides were mounted with 3–5 µm thick sections then deparaffinized with xylene and different grades of ethanol. Citrate buffer (pH=6) was used to mediate antigen retrieval, blocking the samples was done using 1% bovine serum albumin at 21 °C for 10 min. The slides incubated with primary antibody Rabbit polyclonal Caspase-3 antibody (ab44976, Abcam, USA) and Rabbit polyclonal to LC3B (ab48394), Abcam, USA) with dilution 1/ 500) at 21 °C for 2 h. For detection of the primary antibody, secondary antibody (goat anti-mouse IgG polyclonal conjugated with biotin) was used at a dilution 1/200 and visualized using an ABC system. The sections were counterstained using Mayer's Hematoxylin and DPX was used as mounting medium. Negative control sections were done by using phosphate buffer saline instead of primary antibody. Slides were photographed using Olympus® digital camera installed on Olympus® microscope with 1/2 X photo adaptor. Images were transferred to be analyzed on Intel® Core I3® based computer using Video Test Morphology® software (Russia) with a specific built-in routine for area and % area measurement

Statistical Analysis

Values were expressed in the form of mean ± SD and SPSS **(SPSS, Sigma Plot Software, Inc, Chicago, IL)** program statistical package for social science **version 17**) was used to do

statistical analysis. Statistical evaluation was done using one-way analysis of variance (ANOVA) followed by Bonferroni pairwise comparisons. Significance was considered when the p-value was ≤ 0.05

Results

Animal survival

No animals died in normal control group, while in cadmium group, 3 animals died and in AG group only 1 animals died.

Effects of AG on kidney functions (creatinine, Na and K)

Table (1) shows the effect of AG administration on serum creatinine (mg/dl), serum Na^+ (mmol/L) and serum K^+ (mmol/L.) levels. AG led to a highly significant decrease in serum creatinine, Na^+ and K^+ levels as compared to cadmium group, (P< 0.001, = 0.002, <0.001 respectively).

Effects of AG on oxidative stress and antioxidant markers in kidney tissues (GSH and MDA) and urinary KIM-1

Table (2) shows the effect of AG administration on renal tissue MDA (nmol/g. renal tissue), GSH (µmol/g. renal tissue) and KIM-1(pg/ml). AG led to a highly significant decrease in renal tissue MDA, and urinary KIM-1 as compared to cadmium group (P = 0.001 and <0.001 respectively), with a highly significant increase in GSH (P< 0.001) as compared to cadmium group.

Table (1): effect of cadmium (5 mg /kg/d by gastric gavage for 4 weeks) and ghrelin administration (3 nmol /animal per day via subcutaneously route for 4 weeks) on serum Na+ level (mmol/L), K+ serum (mmol/L.) and serum creatinine (mg %)

	Control	Cadmium	Ghrelin	P
Na+ (mmol/L.)	146.3500 ± 2	156± 7	140± 7	< 0.001
K+ (mmol/L.)	4.4±0.26	5.1±0.58	3.9± 0.27	< 0.001
Creatinine (mg %)	0.57 ± 0.08	1.1 ± 0.05	0.91± 0.04	< 0.001

P: as compared with cadmium group

Highly significant: P < 0.001

Table (2): effect of cadmium (5 mg /kg/d by gastric gavage for 4 weeks) and ghrelin administration (3 nmol /animal per day via subcutaneously route for 4 weeks) on tissue malondialdehyde (MDA) (nmol/g. renal tissue), GSH (µmol/g. renal tissue) and kidney injury molecule (KIM-1) (pg/ml).

	Control	Cadmium	Ghrelin	P
Malondialdehyde (MDA) (nmol/g. renal tissue)	7.1±1.3	21.7±4.7	12.6±2.9	< 0.001
GSH (µmol/g. renal tissue)	125.42±15	51±8	88 ±7	< 0.001
KIM-1 (pg/ml)	705 ±211	3062±790	1179±385	< 0.001

P: as compared with cadmium group

Highly significant: P < 0.001

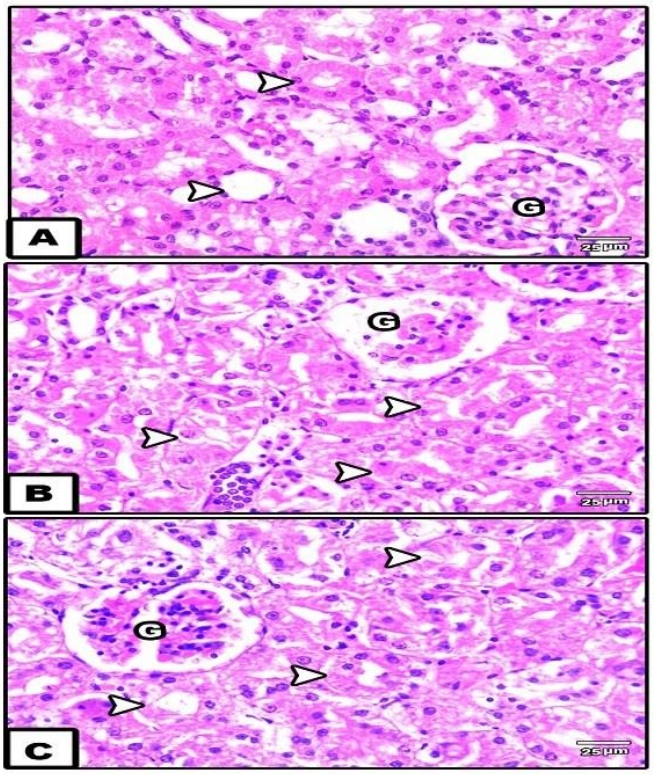

Figure (1): Ghrelin administration attenuated renal injury induced by cadmium. A) H&E staining of the control group B) H&E staining of the cadmium group: hydropic swelling and hypertrophy of proximal tubular cells, as compared with control C) H&E staining of the ghrelin-treated group.

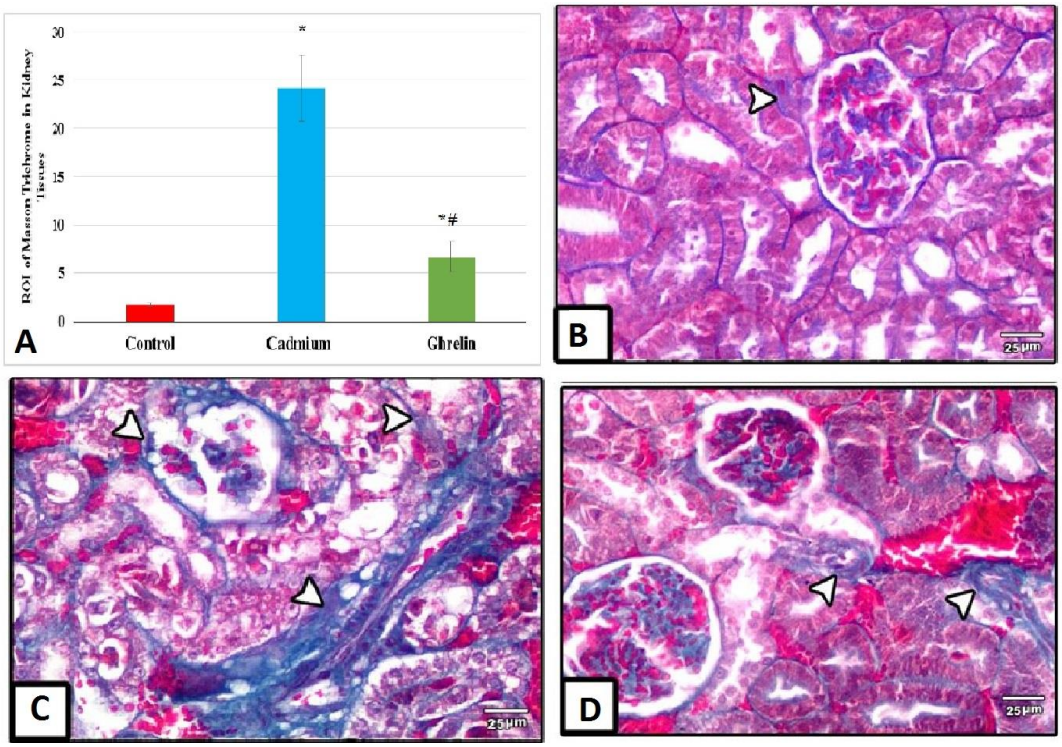

Figure (2): Effect of ghrelin on renal fibrosis by Masson Trichrome. A) ROI of Masson Trichrome staining in kidney tissues. Kidney specimens showing B) no staining with Masson Trichrome i.e. absence of fibrosis (control group), C) mild to moderate fibrosis with Masson Trichrome (blue colour) (Cadmium group) and D) minimal fibrosis stained with Masson Trichrome (blue colour) (ghrelin group).

* Significant vs control group, # significant vs Cadmium group

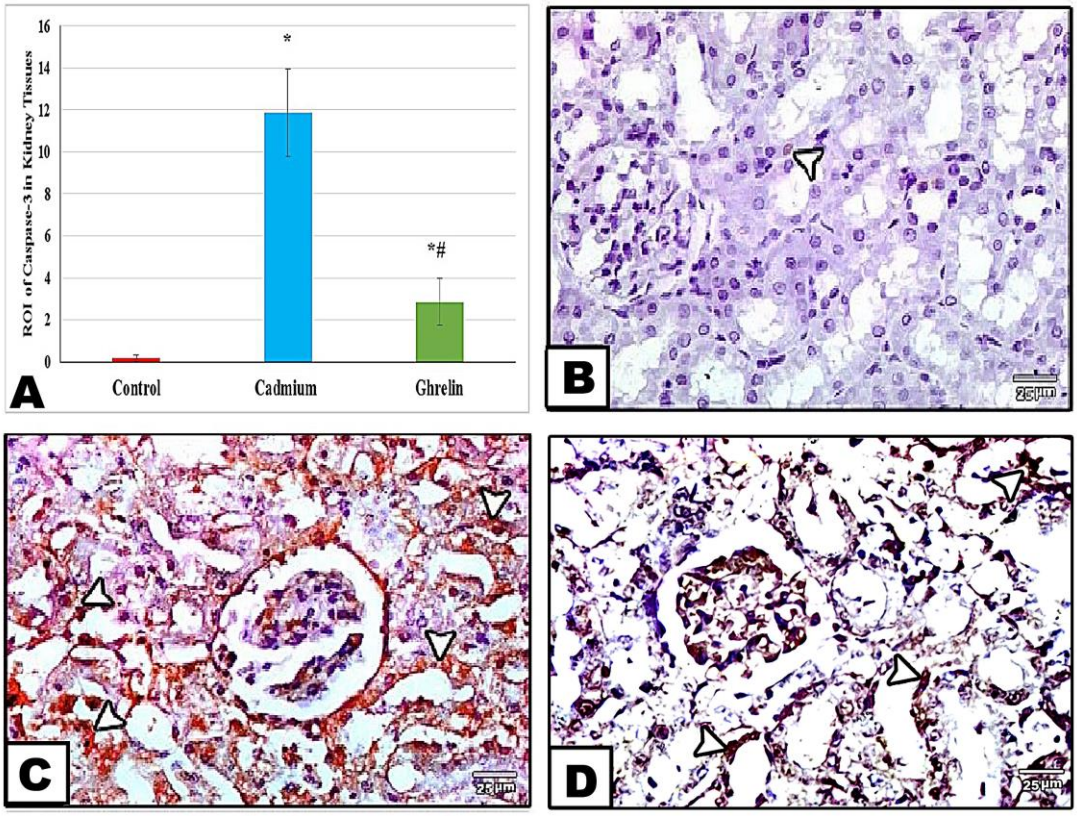

Figure (3): Effect of ghrelin on caspase-3 expression by immunostaining. A) ROI of caspase-3 staining in kidney tissues. Kidney specimens showing B) nearly no staining with caspase-3 i.e. absence of apoptosis (control group), C) extensive expression with caspase-3 (Cadmium group) and D) minimal expression of caspase-3 (ghrelin group). * Significant vs control group, # significant vs Cadmium group.

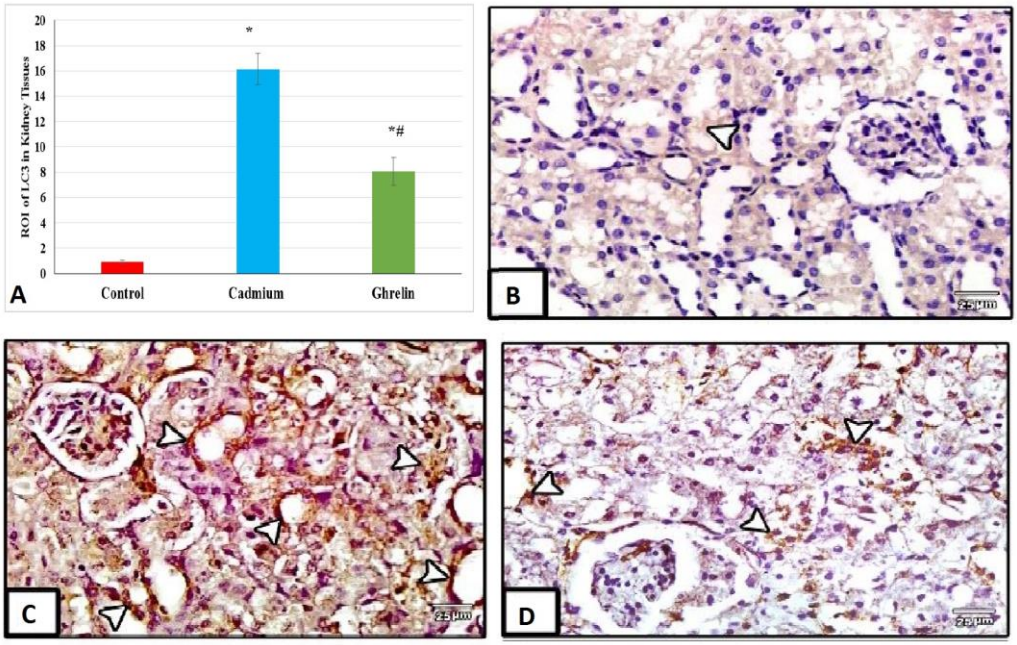

Figure (4): Effect of ghrelin on the expression of autophagy marker (LC3 protein) A) ROI of LC3 expression in kidney tissues. Kidney specimens showing B) minimal LC3 expression in control group, C) increased LC3 level with punctuate vesicular staining and D) significant decrease in LC3 expression in Ghrelin group.

* Significant vs control group, # significant vs Cadmium group

Effects of AG on kidney morphology

(Figure 1B) shows evidence of distorted renal corpuscles with atrophic glomerulus, severe cloudy swelling of the proximal convoluted tubule. This was in contrast to the normal control group (Figure 1A) that shows intact renal architecture. (Figure 1C) showed an improvement in the renal histopathology of the rats when compared with the cadmium group.

Effects of AG on kidney fibrosis

(Figure 2C) showed that chronic Cd exposure for 4 weeks resulted in ECM deposition and fibrosis. This was in contrast to the normal control group (Figure 2B), while (Figure 2D) revealed that AG suppressed the renal collagen deposition showing minimal fibrosis.

Effects of AG on caspase-3 and LC3 expression

(Figures 3B and 4B) shows that there are a few positive cells (brown stain) in the normal control group, while (Figures 3C and 4C) revealed that the positive cells were markedly increased in the cadmium group as demonstrated by increase area and density of positive immunoreaction for caspase-3 and LC3 respectively when compared to normal control group. While (Figure 3D and 4D) revealed that AG significantly inhibit apoptosis and autophagy as demonstrated by a decrease in the area and density of positive immunoreaction for caspase-3 and LC3 respectively when compared to cadmium group.

Discussion

The main findings in the present study include a) Cd administration caused impairment of kidney function and morphology, interstitial fibrosis, enhanced renal oxidative stress, upregulation of LC3 and caspase 3 and b) treatment with ghrelin caused significant improvement in renal functions and morphology, fibrosis which was associated with downregulation of renal oxidative stress ,caspase-3 and LC3 .

In the present study, we found that Cd intoxication induced renal damages as shown by significant increase in kidney function tests (serum creatinine and serum K^+) and deterioration of renal morphology. Histopathological examination showed renal tubular damage in the form of hydropic swelling and hypertrophy of proximal tubular cells, which was associated with interstitial fibrosis. These findings confirm that the induction of this chronic renal model in this current study was successful. On the other hand, coadminsitration of ghrelin with Cd caused significant improvement in kidney functions and morphology as well as fibrosis compared to Cd group suggesting renoprotective effect for ghrelin in Cd-induced nephrotoxicity.

In the current work, to assess the degree of damage of tubules and tubulointerstitial region, kidney injury molecule -1 (Kim-1) level in urine was measured, and it was found that there was a significant elevation in the urinary levels of KIM-1 in Cd group compared to normal control group suggesting that KIM-1 is a good biochemical marker of renal insult induced by Cd. In consistence with this finding, Pennemans, et al., (23) and Ruangyuttikarn, et al., (24) who reported that Kim-1 is considered as a novel urinary biomarker of cadmium induced nephrotoxicity in experimental rats. Moreover, we found a

significant attenuation in KIM-1 level in ghrelin treated group.

Cd is a highly toxic environmental contaminant that stimulates the generation of reactive oxygen species (ROS) (37). These ROS subsequently causes cell membrane and organelles lipids peroxidations that eventually form malondialdehyde (MDA). Thus, measurement of MDA can be used to assess lipid peroxidation. In the present study, the MDA content in renal tissue increased significantly by Cd administration as compared to the control group ($P < 0.001$). This finding is in agreement with several reports demonstrating the induction of oxidative stress in tissues by Cd through stimulation of lipid peroxidation (15, 38). Another finding in the present study that in Cd group the increase in renal tissue MDA, was concomitant with a significant decrease in renal tissue GSH suggesting consumption of this antioxidant by excessive reactive oxygen species. This can be attributed also, to chelation of GSH thiol group by Cd (39, 40, 41). Also, in the present study, treatment with ghrelin reversed significantly the Cd-induced decrease of reduced GSH levels suggesting antioxidant role for ghrelin in Cd-induced renal toxicity. These findings regarding oxidative stress are in line with Singhal et al. (40) who reported that depletion of GSH in renal tissue enhanced Cd toxicity and with many investigators who reported that the elevation of tissue GSH levels protected against Cd induces organ toxicity and relevant injury (42, 43)

Caspase-3 plays a crucial role in the execution phase of cellular apoptosis, which could be activated by intrinsic and extrinsic apoptotic pathways. Caspase3 zymogen has no activity until cleaved into 12-kDa and 17-kDa subunits, which are considered to be apoptotic markers (44). The role of apoptosis in Cd-induced renal injury was demonstrated in different animal models (30, 45, 46). The present study showed similar findings, we demonstrated a significant increase in caspase-3 level in renal tissue image analysis when compared to normal rats. This finding is in agreement with Kondoh et al., (47) who demonstrated that Cd induces cytochrome c release from mitochondria through generation of reactive oxygen species, leading to apoptosis via the activation of the caspase 3 and 9 cascade. Moreover, treatment with ghrelin decreased apoptosis significantly. These findings suggest the role of apoptosis in cadmium-induced renal injury and possible anti-apoptotic effects for ghrelin in this renal injury model.

Several studies reported that ghrelin inhibits apoptosis in many experimental models in different tissues: in PC12 cells through upregulation of heat-shock protein 70 (48), in pancreatic beta cell line, through mitogen-activated protein kinase/phosphoinositide 3-kinase pathways (49) and in lactotrophs apoptosis in the pituitary of diabetic rats (50). Regarding the kidney, Almasi et al., (29) reported the anti-apoptotic effect of ghrelin in kidney tissue in a rat model of chronic hypoxia.

Autophagy mainly acts as a cell survival mechanism. However, excessive autophagy may also contribute to cell death called type II programmed cell death suggesting that autophagy is a double-edged sword. Autophagy has been determined necessary for Cd-induced

hepatotoxicity, testicular injury and mesenchymal stem cells toxicity (51, 52). There is also a mounting evidence that implicates the dysfunction of autophagy process in major neurodegenerative disorders, such as Parkinson's disease, Alzheimer's disease and Huntington's disease (53).

The last objective in the current work was to explore a way of protecting renal function against Cd intoxication through autophagy modulation. The most noteworthy finding of the present study is that there was significant increase of LC3 protein expression in cadmium groups when compared to normal control group. These results suggested that the sustained induction of autophagy did participate in Cd induced renal damage. In the present work, autophagy induction has been postulated to result from oxidative stress and reactive oxygen species; this has been confirmed in the present study by finding significant positive correlation between autophagy and MDA level which is consistent with many investigators who mentioned that reactive oxygen species (ROS) have been considered to be important autophagic activators (54, 55).

There is a controversy regarding the effect of ghrelin on autophagy, some research groups demonstrated that ghrelin induces autophagy (56,57,58) , on the other side others reported the ghrelin inhibited excessive autophagy (59, 30), In the current work, we found significant decrease in LC3 protein expression in ghrelin group when compared to cadmium group, indicating downregulation of autophagy process by ghrelin. In an experimental rat glaucoma model, ghrelin was reported to inhibit neuronal autophagy and apoptosis (30). Down regulation of autophagy by ghrelin could be attributed also to ghrelin antioxidant effect. This is in line with Dong et al., (60) who revealed that antioxidant ascorbic acid exerts neuroprotective function by inhibiting oxidative stress and autophagy in an animal model of seizures, , and, so, according to this speculation; inhibition of autophagy by ghrelin is beneficial which is demonstrated in the present study.

In conclusion, ghrelin attenuates cadmium-induced renal injury by inhibition of oxidative stress with subsequent downregulation of autophagy and caspase-3 in renal tissue.To the best of our knowledge this is the first study that discussed that ghrelin effect on Cd-induced renal damage. This study added positive new insights for understanding the protective nature of ghrelin against Cd induced renal deficits. However, further studies are needed to understand the detailed mechanisms of activation and regulation of Cd-induced autophagy and its role in cell fate to develop novel strategies that depends mainly on protective autophagy for prevention and therapy of chronic Cd renal failure.

Conflict of interest: no conflict of interest

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