ONLINE FIRST

ISSN: 0015-5659

e-ISSN: 1644-3284

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DOI: 10.5603/FM.a2019.0126

Article type: ORIGINAL ARTICLES

Submitted: 2019-08-07

Accepted: 2019-11-03

Published online: 2019-11-26

Cadmium-induced adrenal cortical autophagy in rats: possible modulation by sildenafil

Sildenafil in cadmium-induced adrenal cortical autophagy

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Abstract

Background: The link between autophagy, inflammatory bowel disease, ischemic injury and cancer had been established. Reasonable evidence is available for Cadmium (Cd) to be related to certain cancers. Sildenafil had been investigated to modulate oxidative stress mechanisms. Aim of work: is to investigate Cadmium-induced adrenal cortical autophagy and to declare possible modulation by sildenafil.

Material and methods: Twenty four Wistar rats weighing 150 -200gm were randomly and equally assigned into: Control group, Sildenafil (20mg/kg/day orally) exposed group, Cadmium group (Cd chloride 1mg/kg/day Sc), Cadmium + Sildenafil group (rats received Cd concomitant with Sildenafil). Euthanasia was done 4 weeks from the beginning of experiment; adrenal glands were subjected to biochemical, histological, ultrastructural and immunnohistochemical assessment.

Results: Control and Sildenafil exposed groups exhibited nearly similar results. Cd had produced adrenal cortical apoptosis and ultrastructural derangement of cell organelles. Cd-induced autophagy was detected by ultrastructural abundance of enlarged lysosomes and significant ($p<0.05$) increase in the optical density of LAMP2 (Lysosomal associated membrane protein2) immunoexpression. Sildenafil taken with Cd had decreased adrenal cortical autophagy, significantly modulated the adrenal gland Superoxide dismutase (SOD) and Malondialdehyde (MDA) compared to Cd group. Also, the optical density of NF-κb and Caspase3 immunoexpression was significantly decreased in Cd+Sildenafil compared to Cd group.

Conclusions: Cadmium might induce adrenal cortical autophagy in rats and Sildenafil might show an ameliorating effect probably through enhancement of antioxidant defense mechanism and modulation of NF-κb.
Key words: autophagy, cadmium, adrenal, sildenafil rats

INTRODUCTION

Cadmium is found in solders, used in batteries and as alloy in metal platings and coatings. Reasonable evidence is available for Cadmium to be associated with prostatic cancer [15]. Oxidative stress–mediated cell death and oxygen free radicals (OFRs) production had been suggested to mediate Cadmium-induced cellular toxicity [22].

In ischemic injury and some types of myopathies, extensive autophagy was noticed. Also, in inflammatory bowel disease, polymorphisms in a gene involved in autophagy have been associated. Furthermore, the link between autophagy and cancer had been established [16]. Autophagy, a shuttling of senescent organelles and large protein complexes into lysosomes, is a survival mechanism in times of nutrient deprivation, by which the starved cell can live by digesting its own contents and recycling these contents to provide nutrients and energy [13, 16]. Autophagy might progress to cell death if the stimulus is more severe despite being a mean of avoiding cell death, or the cell metabolic pathways may switch to apoptosis [4].

Sildenafil is a competitive reversible inhibitor of phosphodiesterase 5 (PDE5) that prevents the catabolism of cGMP, prolonging its actions and promoting vasodilation increasing the blood flow to the penis, leading to an erection. Sildenafil is the only agent of PDE5 inhibitors currently approved for the treatment of pulmonary hypertension [27]. In rat models of stroke, sildenafil had been reported to increase brain levels of cGMP. Furthermore, it enhanced the cerebral blood flow level in the hypoperfused region, increased angiogenesis and neurogenesis which can rapidly enhance functional recovery [30, 17]. High dose of sildenafil had been proved to protect against carbon tetrachloride-induced adverse renal changes by modulation of redox homeostasis in rats [1]. The current study was carried out to investigate Cadmium-induced adrenal cortical autophagy and to declare possible modulation by sildenafil.

MATERIALS AND METHODS

Animals

The current work had followed all ethics of animal research and had taken approval from the Institutional Animal Care and Use Committee of Cairo University (CU-IACUC) No CU/III/F/39/19. The present study was carried out on twenty four male Wistar rats weighing 150 -200, housed in the Animal House, Faculty of Medicine, Cairo University under standard laboratory and environmental conditions with free access to food and water at a temperature of (20±2ºC) with a natural 12-h light/dark cycle.

Chemicals

1. **Cadmium**: Cadmiun Chloride salt was purchased from Sigma Chemical Co. (St. Luis, Missouri, USA). After dissolving in saline, it was offered every day by SC injection (1mg/kg /day) **[2].**.
2. **Sildenafil**: Sildenafil tablets (Viagra 50mg, Pfizer, Egypt) were dissolved in 10 ml saline and given at a dose of (20 mg/kg) offered every day by oral gavage **[1].**

Experimental design

The rats were randomly assigned into four equal groups (6 rats each) as follows:

Control group (received oral saline), **Sildenafil exposed group** (received oral Sildenafil), **Cadmium group** (received Cadmium), **Cd+Sildenafil group** (received Cadmium concomitant with Sildenafil.

All animals were weighed just before sacrification. They were euthanized after four weeks from the beginning of the experiment by Pentobarbital 150mg/kg IP. After euthanasia of animals, the two adrenal glands of each animal were immediately removed, weighed, half of one adrenal was used for biochemical assessment, the other half underwent paraffin blocking for histological evaluation. The other Adrenal gland had undergone EM processing.

a- Biochemical analysis:

Adrenal gland homogenates were centrifuged at 4000 r.p.m. for 20 minutes and refrigerated at 4 °C. The supernatant was used for estimation of the quantitative activities of **(MDA)** and **(SOD)** as described in **Adeyanju et al. 2018 [1]**.

b- Histological and Immunohistochemical study:

Adrenal gland sections of 5 μm thickness were Haematoxylin & Eosin (H &E) stained. Immunohistochemical study **[25]**, utilizing Dako automated technique, was done to deparaffinized sections for staining with Lysosomal associated membrane protein2 (LAMP2), NF-κB, and Caspase3. Primary antibodies used were LAMP2 (Rabbit polyclonal, Genetex, USA), NF-κB p65 (Rabbit polyclonal, ThermoFisher, USA), Caspase3 (Rabbit polyclonal, Santa Cruz, USA). Dako EnVision Flex/HRP was utilized as secondary antibody and Leica ICC50 microscope was used for photographing all histological and immunosistochemical sections.

For ultrastructural study **[12]**, adrenal glands were fixed for 2 hours in 2.5% glutaraldehyde buffered with 0.1 M cacodylate at pH 7.2, 1% osmium tetroxide postfixed, alcohol dehydrated, embedded in epoxy resin mixture after immersion in propylene oxide. Semi-thin sections of about 1μm thickness 1% toluidine blue stained, and then photographed with Leica ICC50 light microscope. Ultrathin sections (80–90 nm) were obtained using an LKB ultratome, and stained by uranyl acetate and lead citrate. The ultrastructural photographing was done using a transmission electron microscope (Joel Jem 1400, Germany), Faculty of Agriculture, Cairo University, Egypt.

c- Histomorphometric study:

The software Leica Quin 500, Germany was utilized to assess the optical density of LAMP2, NF-κB and Caspase3 immuno-reaction in a standard measuring frame using a magnification x 400. Values were presented as a mean and standard deviation and statistically analyzed.

Statistical analysis

Morphometric, biochemical and histomorphometric data were statistically analyzed utilizing SPSS version 25.0 (IBM Corporation, Somers, NY, USA) statistical software. They were expressed as means ± Standard Deviation (SD). One-way analysis of variance (ANOVA) had been performed, then posthoc Tukey test was utilized to compare between groups.

RESULTS

Clinical data, Body and adrenal gland weight (Table 1)

No mortality had been reported among rats. The body weight of Cd group was significantly decreased compared to the control group. No significant difference ($p>0.05$) among the groups was noticed in adrenal gland weight.

Biochemical results (Fig. 1)

MDA of Cd group was significantly higher (238 %) than that of control group. MDA of Cadmium + Sildenafil was non significantly higher (61%) than that of control group, but was significantly lower (52 %) than that of Cd group. SOD of Cd group was significantly lower (66 %) than that of control group. SOD of Cd + Sildenafil was significantly lower (33 %) than that of control group, but significantly higher (33 %) than that of Cd group.

Histological results:

By H&E (Figs. 2, 3) staining, Control and sildenafil groups exhibited regular cells in all zones with dilated sinusoids sinusoids in Zona Reticularis (ZR) of sildenafil group. Cd group showed disturbed architecture, wide inercellular spaces, cytoplasmic vaculations and frequent apoptosis as well karyolysis. Cd+Sildenafil group showed slight vaculation in Zona glomerulosa (ZG), minimal apoptosis in Zona fasciculata (ZF) and lipofuscin pigments in ZR. Toluidine blue staining **(Fig. 4)** of the adrenal cortex of the control and Sildenafil groups was similar. Cd group ZG and ZF sowed many large lipid droplets compressing neighboring nuclei while Cd ZR revealed cytoplasmic rarefaction with marked nuclear pleomorphism. Cd+Sildenafil group exhibited slight nuclear degeneration in ZF.

EM results (Figs. 5, 6)

Control and Sildenafil groups exhibited similar findings. Cd group ZG cells appeared with dilated smooth endoplasmic reticulum while Cd ZF spongiocytes and ZR cells exhibited shrunken nuclei, dilatation of smooth endoplasmic reticulum, loss of mitochondrial cristae and perinuclar swelling as well as abundant enlarged lysosomes containing many digested organelles. Cd+Sildenafil group revealed cells with intact mitochondria, many lipid droplets and few lysosomes in ZF.

Immunohistochemical results

By LAMP2 immunostaining (Fig. 7), Control and Sildenafil groups exhibited similar results with mild reaction in all zones of the cortex. Cd group showed markedly increased LAMP2 while Cd+Sildenafil group showed mild to moderate reaction.

By NF-kb immunostaining (Fig. 8), Control and Sildenafil groups exhibited similar results with mild reaction in all zones of the cortex. Cd group showed strong Nf-kb immunoexpression in all zones while Cd+ Sildenafil group showed moderate reaction.

By Caspase3 immunostaining (Fig. 9), Control and Sildenafil groups exhibited similar results with mild reaction in all zones of the cortex. Cd group exhibited increased Caspase3 immunoexpression esp. in ZF while Cd+ Sildenafil group showed decreased reaction.

Histomorphometric results (Fig. 1)

The Optical density of LAMP2, NF-κb and Caspase3 immunoexpression was significantly higher in Cd group (80%, 75%, and 136% respectively) than control group. It was significantly lower (31%, 32%, and 35%) in Cd+ Sildenafil group than Cd group. The Optical density of LAMP2 and NF-κb immunoexpression was non significantly higher (19%, 18% respectively) in Cd+Sildenafil group than the control. The Optical density of Caspase3 immunoexpression was significantly higher (51%) in Cd+Sildenafil group than the control.

DISCUSSION

Cadmium had induced adrenal cortical autophagy in the current work. Concomitant administration of Sildenafil with cadmium had modulated this autophagy and other ultrastructural deleterious effects. The results of this study presented that exposure to cadmium had induced body weight loss in cd group despite no significant change in adrenal gland weight had been detected. Concordantly, Mutsuga et al. (2017) [21] had reported similar finding upon exposure to Aminoglutathimide. The latter authors attributed this slight relative increase in the adrenal weight to lipid droplets accumulation. Adrenal gland MDA and SOD had been modulated by sildenafil as compared to Cd received rats in the current work. This might indicate the antioxidative stress role of Sildenafil. Supporting these

findings, it was reported that Sildenafil has significantly decreased the elevated MDA level in gentamicin-nephrotoxicity [20]. In addition, it was demonstrated that Sildenafil had decreased renal MDA upon exposure to cecal ligation and puncture-induced sepsis in rats [5].

Frequent apoptotic cells were seen in Cd group, while few apoptotic cells were found in Cd+Sildenafil group. This was confirmed by significant reduction in Caspase3 immunoexpression in Cd+Sildenafil group. Supporting these results, Sildenafil had been proved to ameliorate apoptosis in the ischemic myocardium [7]. The mecahanism by which Sildenafil mitigates apoptosis has been suggested by some authors. up-regulation of vascular endothelial growth factor expression [6]) and promoting angiogenesis in the periinfarct region after stroke [17, 9] had been proposed as mechanism of action. Opening of mitochondrial ATP-sensitive potassium (mito-K_{ATP}) channels had been suggested to mediate the cardioprotective effects of sildenafil [7]. Also, activation of protein kinase C and PKG [8] had been early proposed. However, the exact method by which Sildenafil exerts as antiapoptotic is intriguing and warrants further researches.

In the present study Cd group showed increased vacuolation of the ZG and ZF cells in H&E sections together with fatty degeneration in semithin sections. Fatty change, which may be reversible in moderate degrees, is accumulation of lipid droplets due to disruption of ribosomal function and uncoupling of lipid from protein metabolism [4]. Lipofuscin was detected in the current work in ZR of Cd+Sildenafil group. It is present normally in ZR of adrenal gland and is considered as an aging or atrophy insoluble brownish-yellow granular “wear-and-tear pigment” that accumulates in a variety of tissues representing complexes of lipid and protein that are produced by the free radical–catalyzed peroxidation of polyunsaturated lipids of subcellular membranes [16, 19].

Adverse ultrastructural changes in the adrenals of Cd group of this work were partially improved by Sildenafil. Disruption of the mitochondrial cristae could be explained by accumulation of lipid granules caused by impaired steroidogenesis. The enzymes 11-hydroxylase (CYP11) in mitochondria and 17- and 21-hydroxylases (CYP 17 and 21) in the smooth endoplasmic reticulum had been suggested to be commonly disrupted by toxic chemicals [24]. The increased number of enlarged lysosomes containing degraded organelles in Cd group and the significant increase in LAMP2 immunoexpression might indicate Cd-induced autophagy in the

adrenocortical cells. Although not measured in the present work, TEM morphometry for quantification of autophagy has been reported to be rather controversial, and unreliable procedures still continue to be used [14]. The relation between autophagy and cancer had been established. Cd-induced autophagy in the current work might suggest a clue to the link between cadmium and prostatic cancer [23] and further researches are still deserved. Whether autophagy might be good or bad from the vantage point of the tumor, however, stay a matter of debate and active investigation [16]. Modulation of LAMP2 immunexpression by sildenafil in this work is concordant with its ultastrucural regression in the number of enlarged lysosomes induced by cadmium. LAMP-2-positive granules had been reported by Mutsuga et al. (2017) [21] to be increased in the fasciculata cells of Aminoglutethimide (antitumour agent in breast and prostate cancer) reflecting its-induced intracellular mitophagy and lipophagy, which are protein degradation processes for damaged mitochondria and excessively accumulated lipid droplets, respectively [26, 28].

NF-kb immunoexpression had been significantly increased in Cd received rats. Sildenafil had modulated this expression. NF-κB had been considered as an important transcription factor, participates in growth, differentiation, organogenesis, apoptosis, inflammation and immune response [18]. The link between NF-κB and autophagy in the current work might have been established in Cd group. Concordantly, Quinazolinediamine which is an NF-*κ*B inhibitor had been proved to ameliorate both autophagy and apoptosis induced by brefeldin [31]. Agreeing with the results of this work the relation between Sildenafil and NF-κB, it had been proved that Roflumilast, a phosphodiesterase4 inhibitor, might mitigate cadmium-induced renal toxicity via modulation of NF-κB activation and induction of NQO1 in rats [3, 28].

The crosstalk between apoptosis and autophagy in cadmium-induced adrenal cortical injury had been demonstrated in the current work. This crosstalk might be mediated through Caspase3 activation, impairment of antioxidant defense and Nf-kb activation as proved from immunohistochemical and biochemical results of the present work. The latter hypothesis was supported from an earlier work that suggested that this crosstalk was mediated via Caspase cleavage of Beclin 1 [10]. Contradictory to our hypothesis, autophagy had been proved to possess a pivotal role in neuronal antioxidant pathway [11]. Further mechanistic studies are still warranted to investigate this crosstalk.

Clinically implicated from the current work, Cadmium might induce adrenal cortical autophagy and apoptosis. Sildenafil might be valuable in preventing such adverse changes. However, the link between cadmium-induced autophagy, risk of cancer and antioxidant defense system is intriguing and further researches are deserved.

CONCLUSIONS

Cadmium might induce adrenal cortical autophagy in rats and Sildenafil might show an ameliorating effect probably through enhancement of antioxidant defense mechanism and modulation of NF-κb.

Conflicts of interest: All authors had declared no conflicts of interests.
Acknowledgements: All authors had received no funds for the current research.

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Table I. Comparison of the mean body and adrenal gland weight among the different groups

Groups	**Mean body weight Gm ± SD (No of animals in each group=6)**	**Mean adrenal gland weight Gm ± SD (No of animals in each group=6)**
Control	200.5 ± 11.4	0.028 ± 0.004
Sildenafil	200.8 ± 16.	0.027 ± 0.004
Cd	177.0 ± 12.4$^{*,\#}$	0.026 ± 0.006
Cd+Sidenafil	191.7 ± 13.9	0.027 ± 0.005

*, $^{\#}$ = statistically significant compared to Control, Sildenafil exposed, Cd groups respectively

Figure 1. Charts of the mean mean of adrenal gland MDA and SOD as well as the Optical density of LAMP2, NF-kB and Caspas3 immunoreaction among different groups (No of each group=6). *, $^{\#}$, $^{@}$ = statistically significant compared to Control, Sildenafil exposed, Cd groups respectively.

Figure 2. Control and sildenafil groups are showing regular cells in all zones with dilated sinusoids sinusoids **(S)** in ZR of sildenafil group. **(H&E x 400)**

Figure 3. Cd group is showing frequent apoptosis **(arrows)**, frequent karyolysis **(arrow heads),** disturbed architecture, wide inercellular spaces and numerous cytoplasmic vaculations **(V)**. Cd+Sildenafil group is showing slight vaculation in ZG, minimal apoptosis in ZF and lipofuscin **(curved arrows)** pigments in ZR. **(H&E x 400)**

Figure 4. Cd group ZG and ZF are exhibiting many large lipid droplets **(L)** compressing neighboring nuclei **(arrow head)** while Cd ZR is exhibiting cytoplasmic rarefaction with marked nuclear **(arrow heads)** pleomorphism. Cd+Sildenafil group is showing slight nuclear degeneration in ZF. **(Toluidine blue x 1000)**

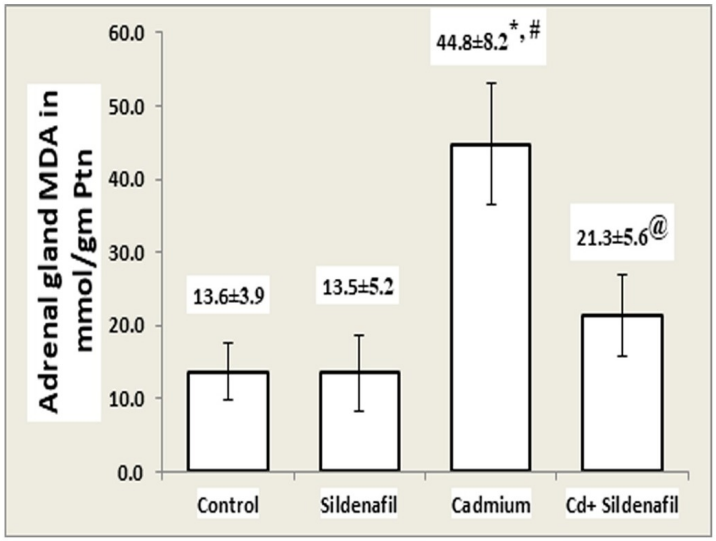
Figure 5. Cd group ZG cells is appearing with dilated smooth endoplasmic reticulum while Cd ZF and ZR cells are exhibiting shrunken nuclei with dilatation of smooth endoplasmic reticulum, loss of mitochondrial **(M)** cristae as well as abundant enlarged lysosomes **(LY)** containing digested organelles. Cd+Sildenafil group appear with many lipid droplets **(L)** and few lysosomes in ZF. **(EM)**

Figure 6. A, B & C: Cd group ZG, ZF & ZR respectively are showing large lipid droplets **(L)** compressing the nucleus, dilatation of smooth endoplasmic reticulum **(SER)**, abundant lysosomes **(LY)**, perinuclar swelling **(arrow heads)** and mitochondrial **(M)** degeneration. **D-** Cd+Sildenafil group ZF appears with abundant mitochondria and lipid droplets. **(EM)**

Figure 7. Cd group is showing strong LAMP2 **(arrows)** immunoexpression while Cd+Sildenafil group is showing mild to moderate reaction. **(LAMP2x 400)**

Figure 8. Cd group is showing strong Nf-kb **(arrows)** immunoexpression while Cd+Sildenafil group is showing moderate reaction. **(Nf-kb x 400)**

Figure 9. Cd group is showing increased Caspase3 **(arrow)** immunoexpression esp. in ZF while Cd+Sildenafil group is showing decreased reaction. **(Caspase3 x 400)**


Adrenal gland MDA in mmol/gm Ptn
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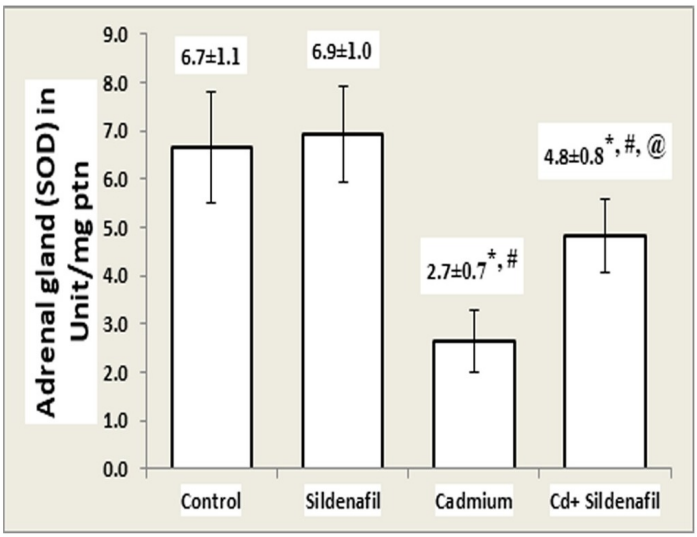
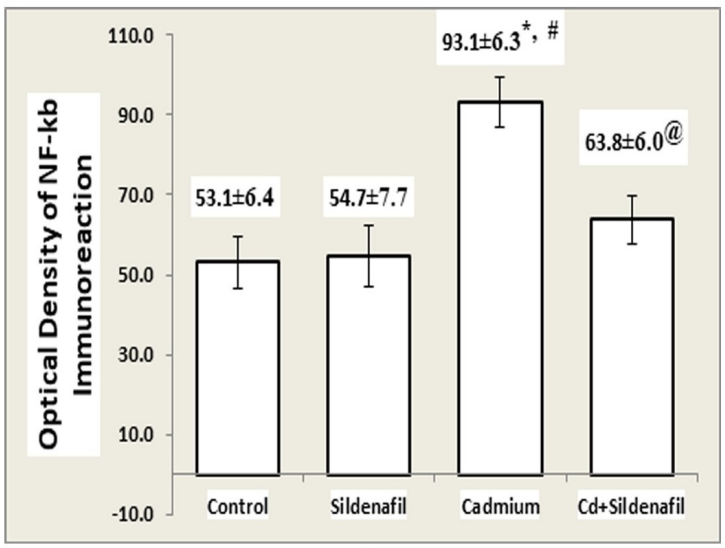
50.0
40.0
30.0
20.0
10.0
0.0
13.6±3.9
13.5±5.2
44.8±8.2*, #
21.3±5.6@
Control
Sildenafil
Cadmium
Cd+ Sildenafil


Adrenal gland (SOD) in Unit/mg ptn
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
6.7±1.1
6.9±1.0
2.7±0.7*, #
4.8±0.8*, #, @
Control
Sildenafil
Cadmium
Cd+ Sildenafil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110.0
90.0
70.0
50.0
30.0
10.0
-10.0
53.1±6.4
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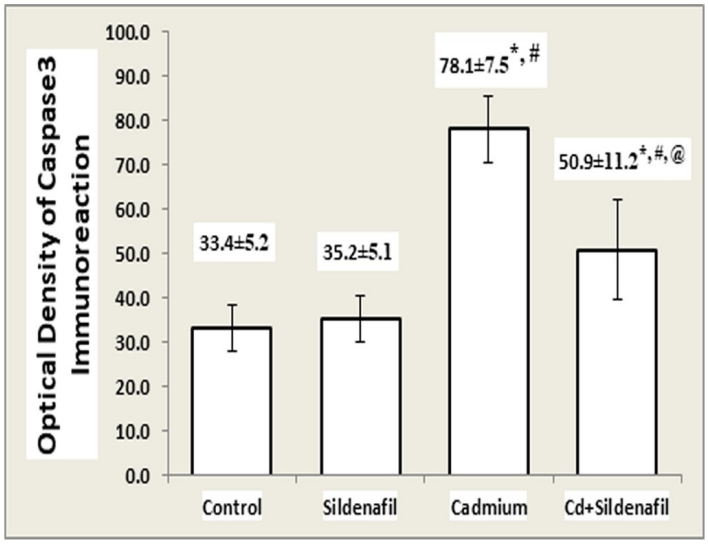
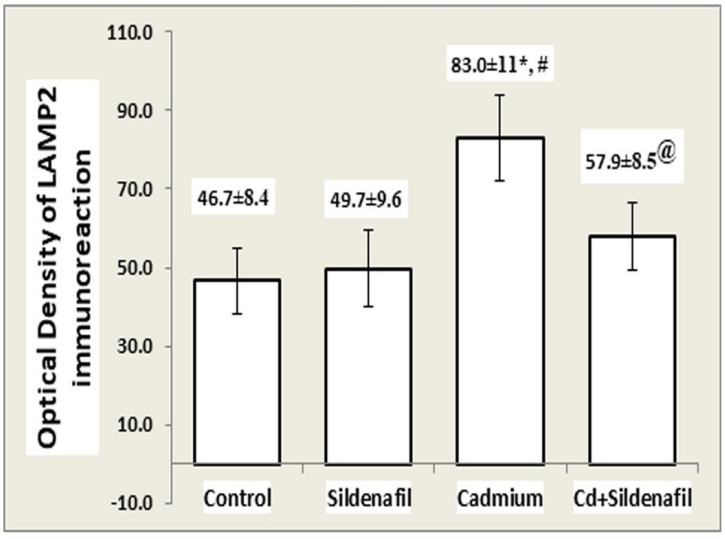
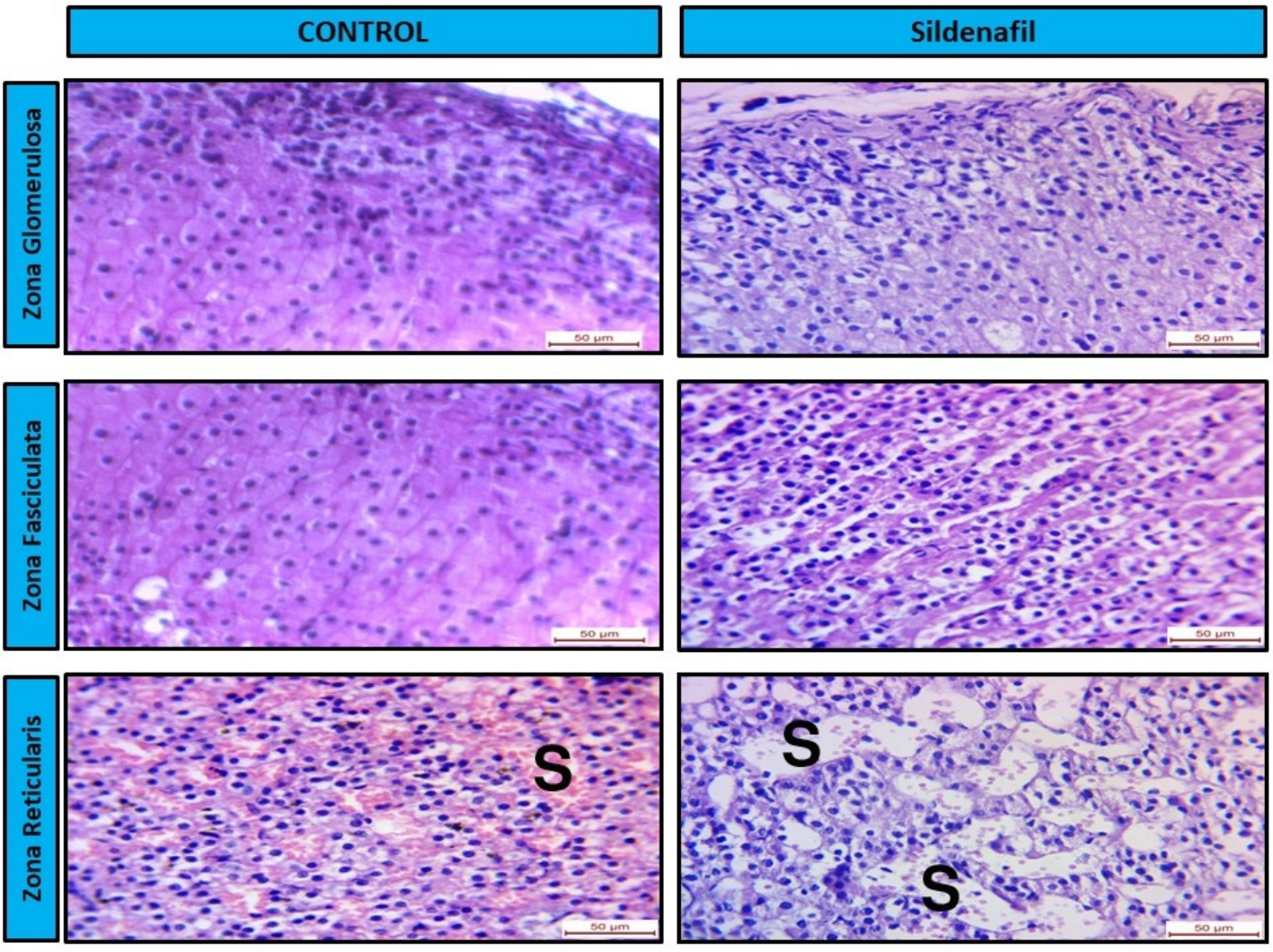
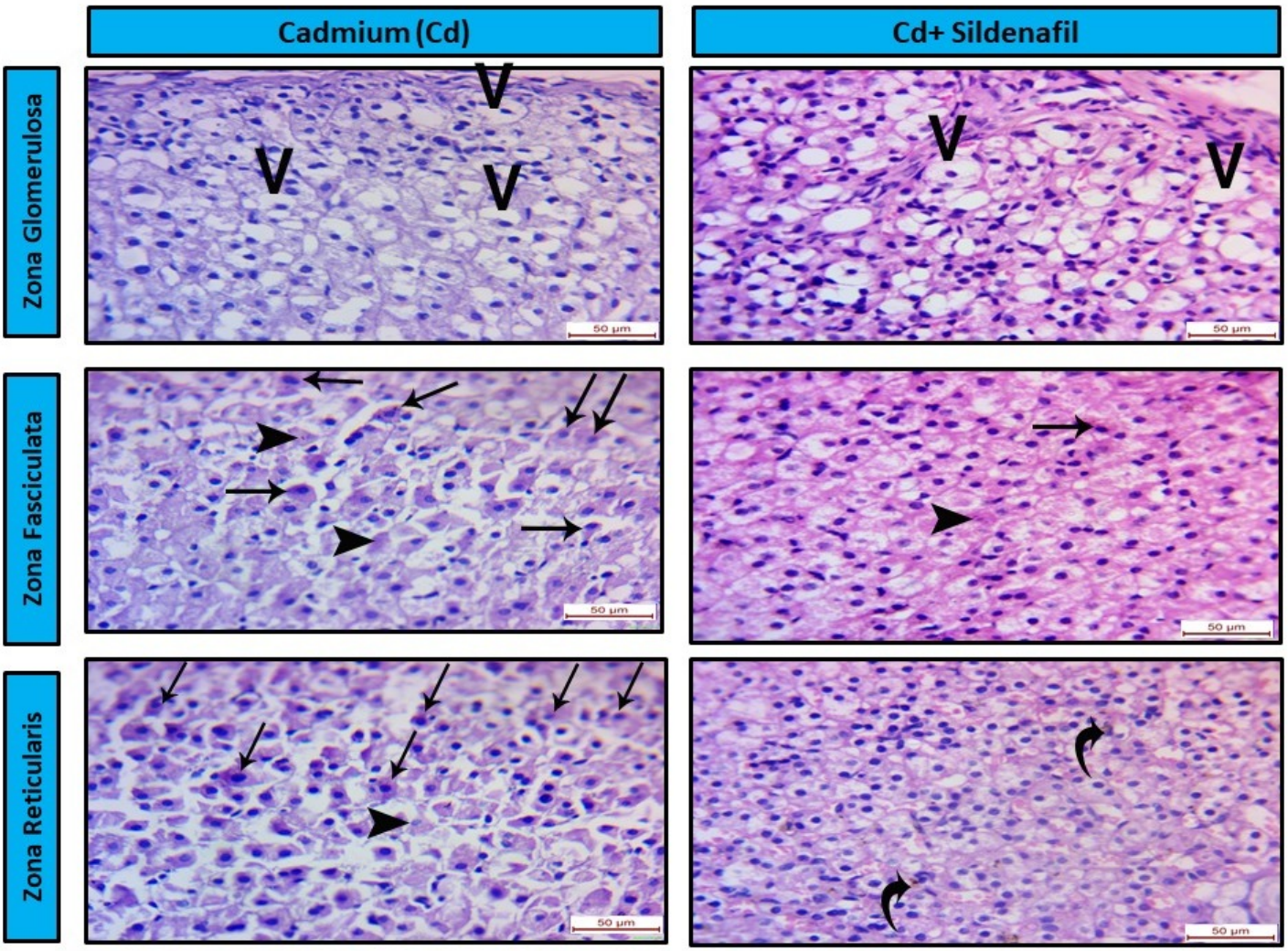
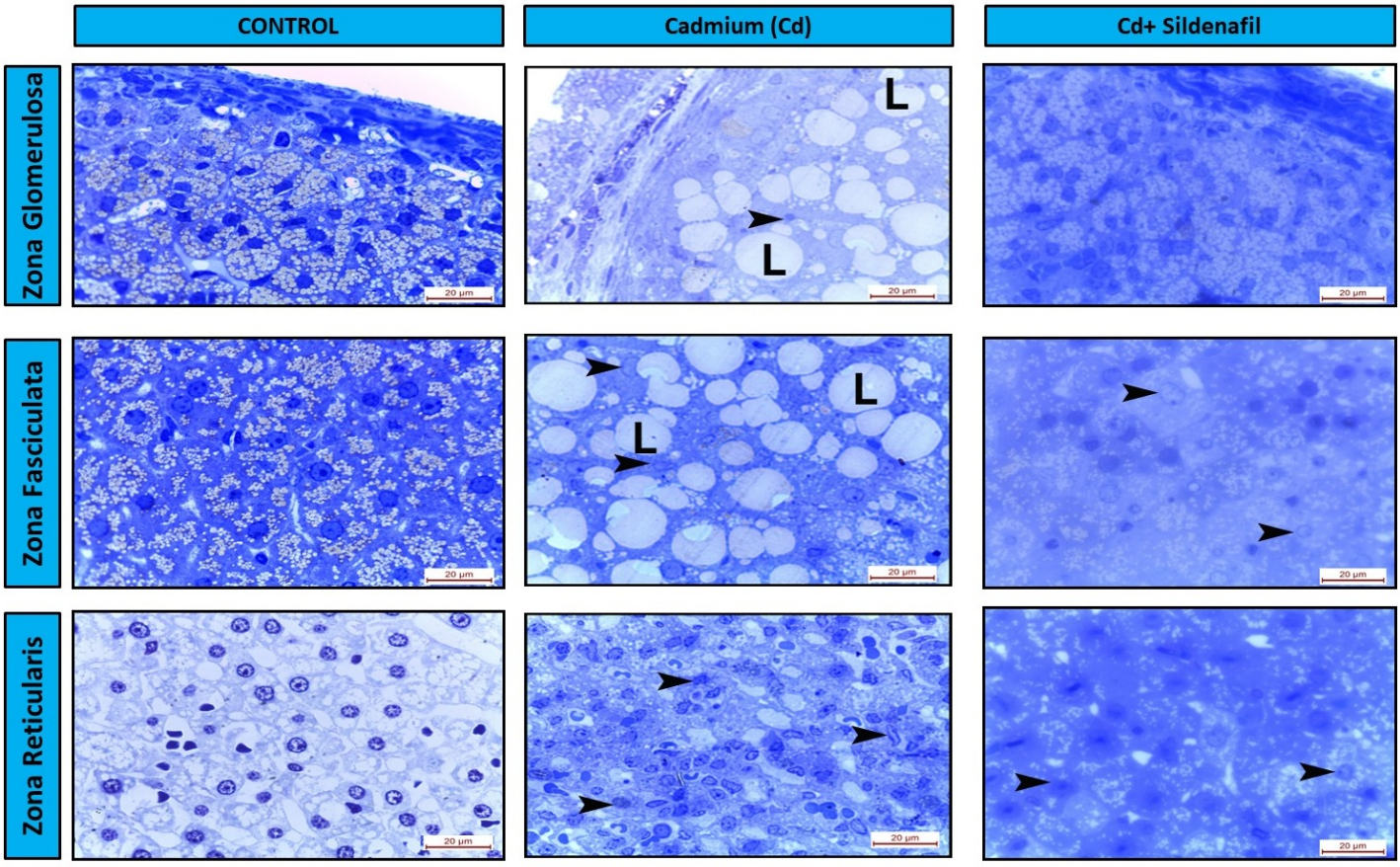
Control
Sildenafil
Cadmium
Cd+Sildenafil

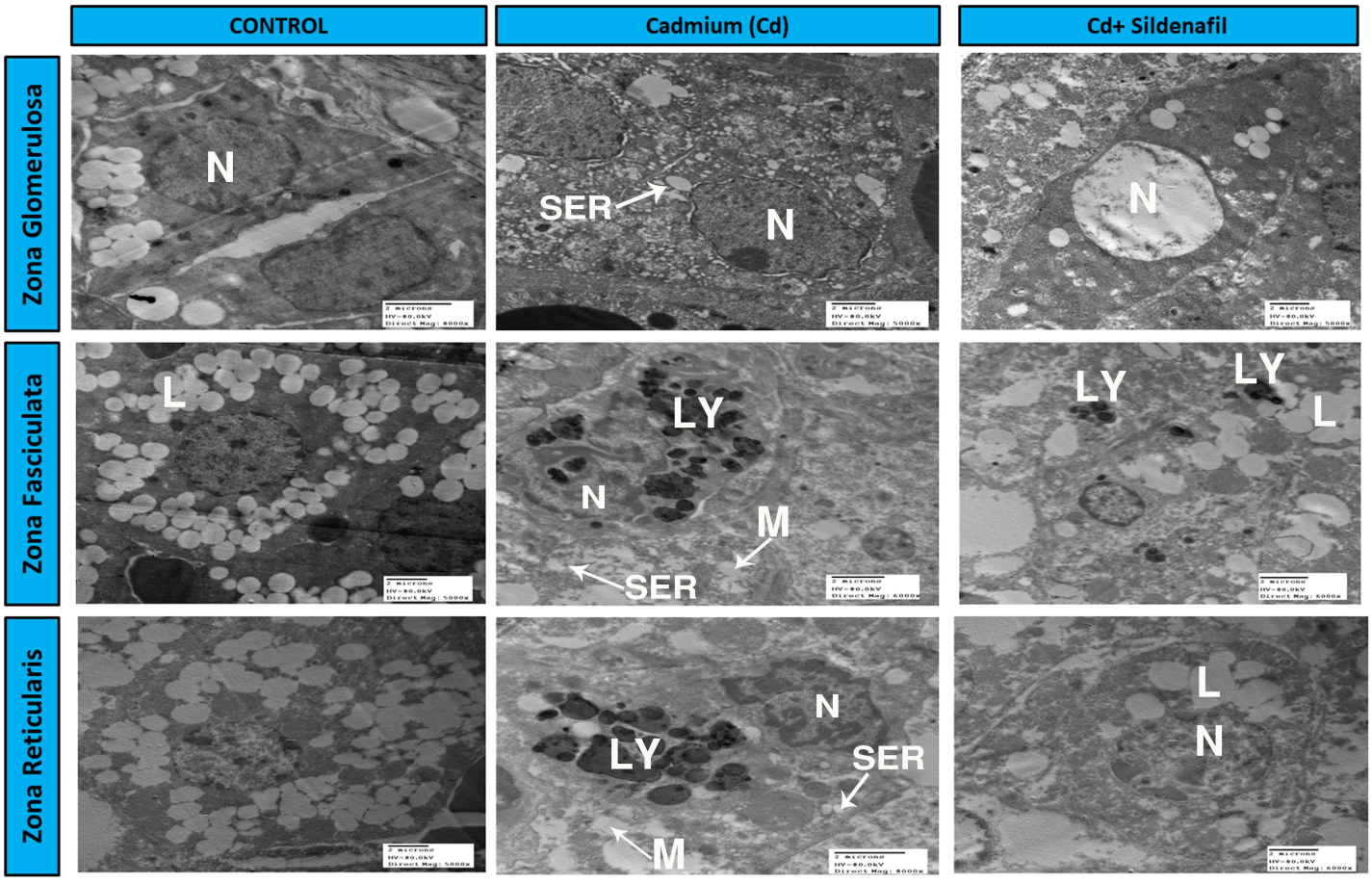

Optical Density of Caspase3 Immunoreaction
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
33.4±5.2
35.2±5.1
78.1±7.5*, #
50.9±11.2*, #, @
Control
Sildenafil
Cadmium
Cd+Sildenafil

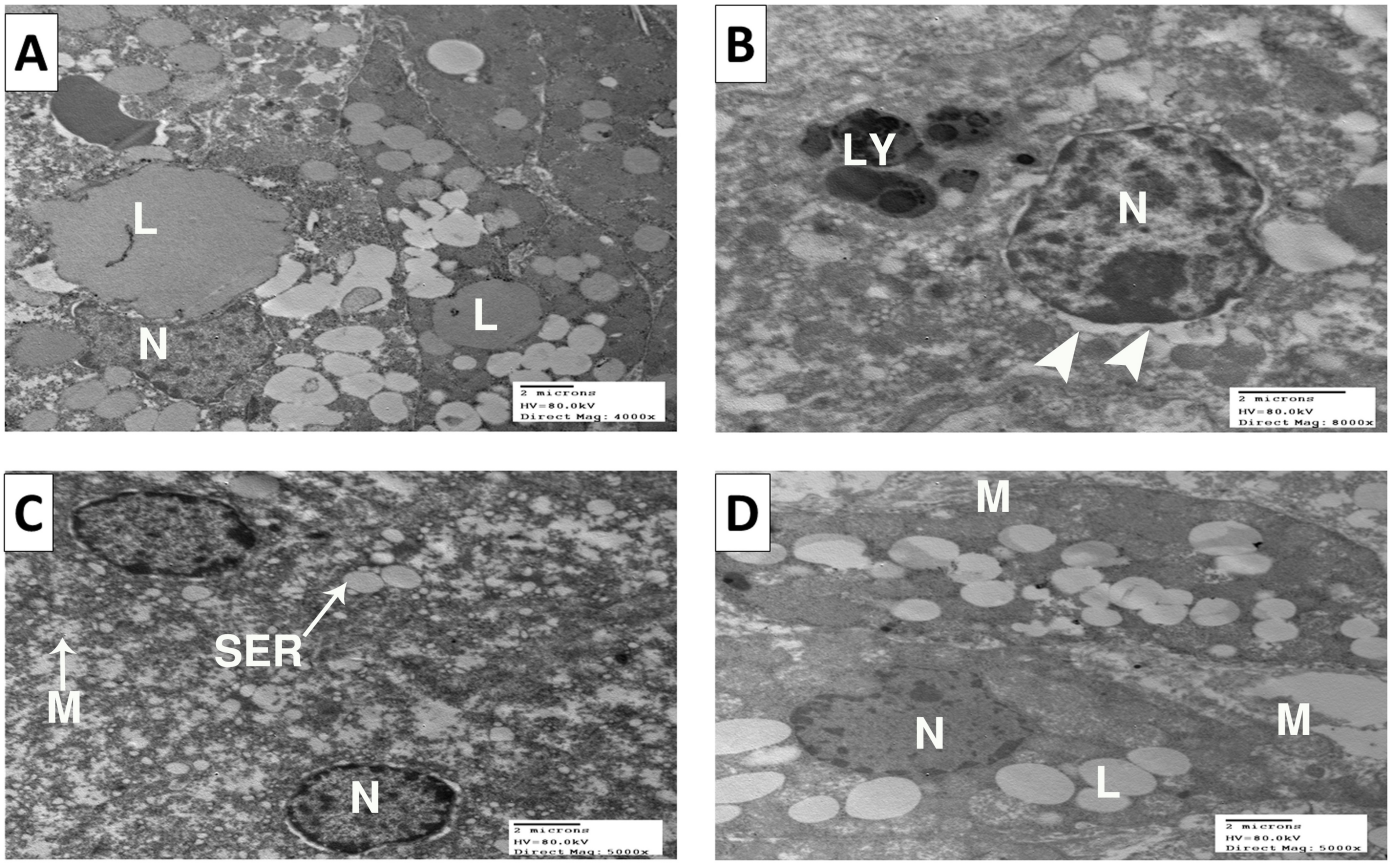

Optical Density of LAMP2 immunoreaction
110.0
90.0
70.0
50.0
30.0
10.0
-10.0
46.7±8.4
49.7±9.6
83.0±11*, #
57.9±8.5@
Control
Sildenafil
Cadmium
Cd+Sildenafil

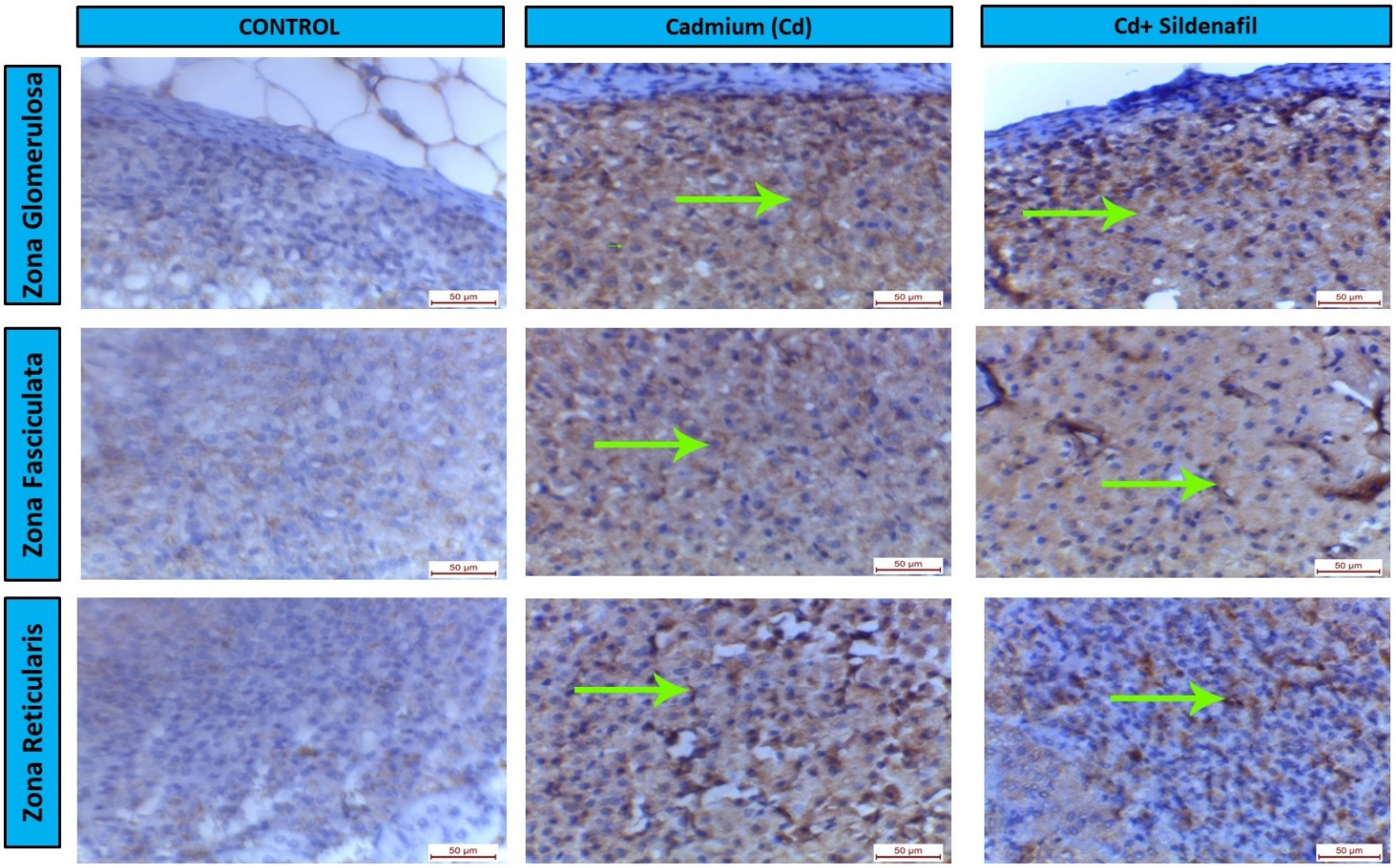

CONTROL
Sildenafil
Zona Glomerulosa
Zona Fasciculata
Zona Reticularis
S
S
S
50 µm
50 µm
50 µm
50 µm
50 µm
50 µm

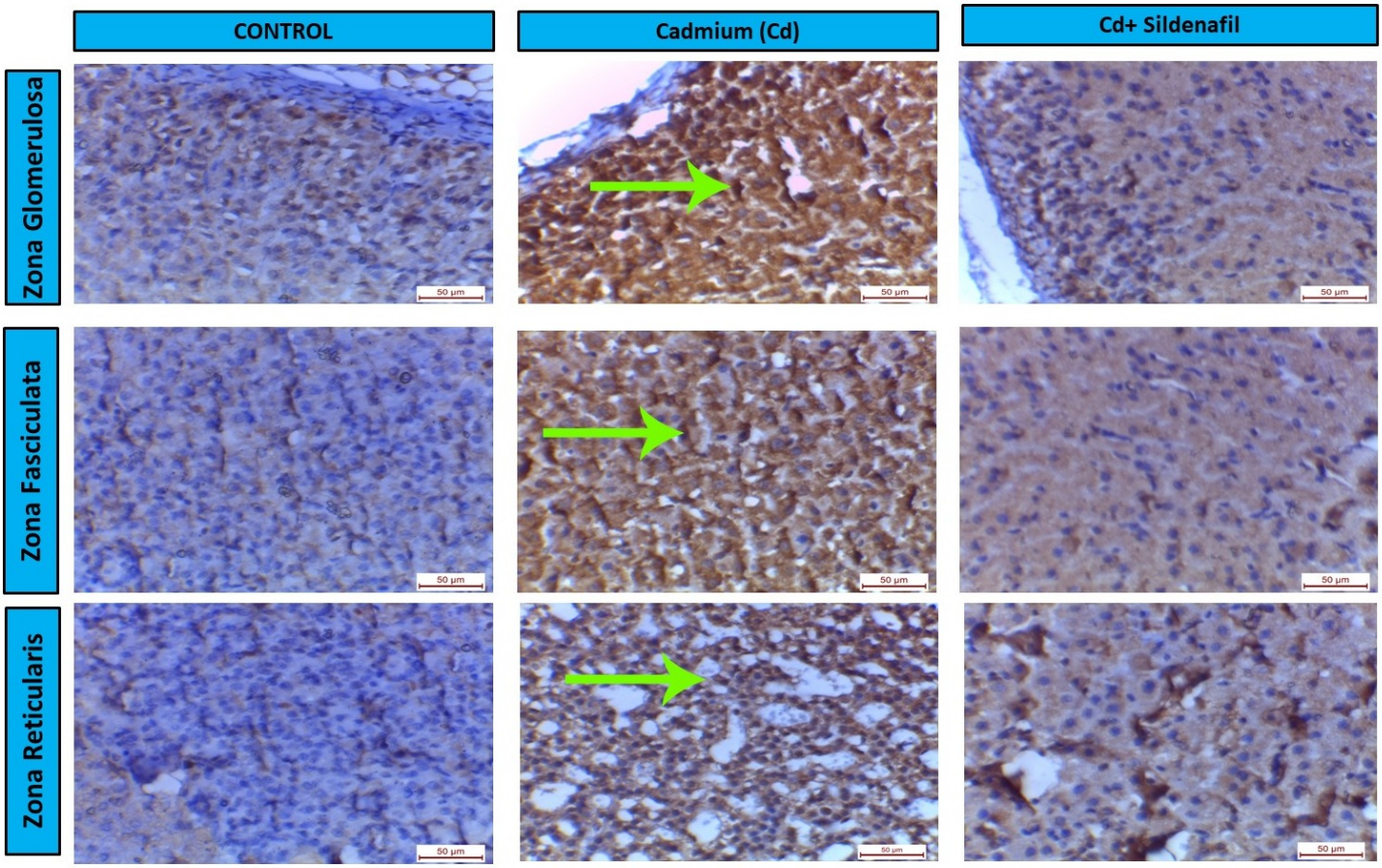

Cadmium (Cd)
Cd+ Sildenafil
Zona Glomerulosa
Zona Fasciculata
Zona Reticularis
V
V
V
V
V
50 µm
50 µm
50 µm
50 µm
50 µm
50 µm

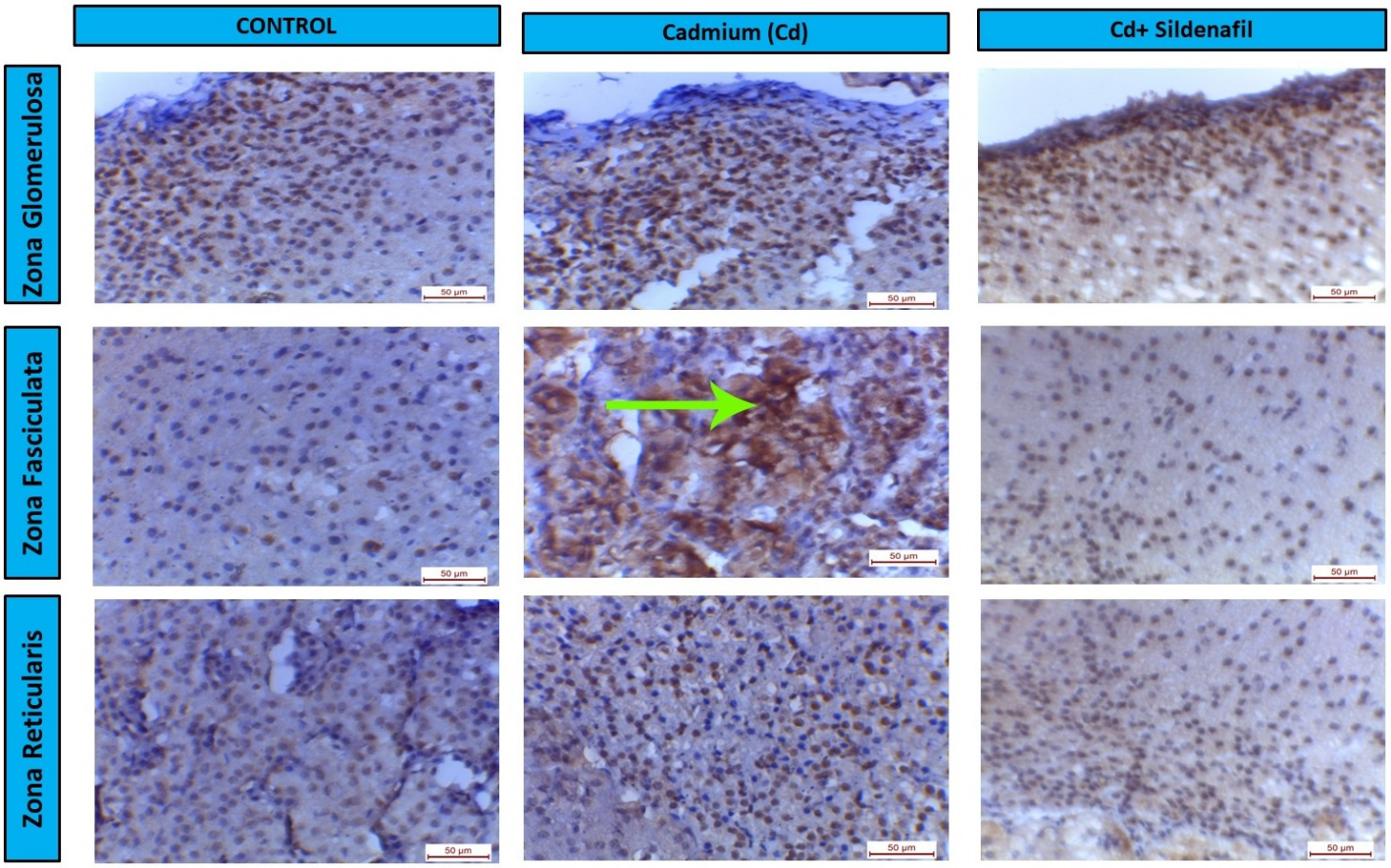

CONTROL
Cadmium (Cd)
Cd+ Sildenafil
Zona Glomerulosa
Zona Fasciculata
Zona Reticularis
L
L
L
L
20 µm
20 µm
20 µm
20 µm
20 µm
20 µm
20 µm
20 µm
20 µm


CONTROL
Cadmium (Cd)
Cd+ Sildenafil
Zona Glomerulosa
Zona Fasciculata
Zona Reticularis
N
SER
N
N
L
LY
N
M
SER
LY
LY
L
N
LY
SER
M
L
N


A
L
N
L
2 microns
HV=80.0kV
Direct Mag: 4000x
B
LY
N
2 microns
HV=80.0kV
Direct Mag: 8000x
C
SER
M
N
2 microns
HV=80.0kV
Direct Mag: 5000x
D
M
N
M
L
2 microns
HV=80.0kV
Direct Mag: 5000x


CONTROL
Cadmium (Cd)
Cd+ Sildenafil
Zona Glomerulosa
Zona Fasciculata
Zona Reticularis
50 µm


CONTROL
Cadmium (Cd)
Cd+ Sildenafil
Zona Glomerulosa
Zona Fasciculata
Zona Reticularis
50 µm


CONTROL
Cadmium (Cd)
Cd+ Sildenafil
Zona Glomerulosa
Zona Fasciculata
Zona Reticularis
50 µm