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RESEARCH ARTICLE

Impacts of Graded Doses of Zincsulfate on Caspase-3 Enzyme Level and Myocardial Apoptosis in Mitoxantrone-induced Cardiotoxicity in Rats

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Abstract

Zinc is regarded as a necessary mineral for cell division and the production of DNA and protein; furthermore, such mineral has a main role in alleviating cardiovascular diseases and may have protective effect in coronary artery disease. Mitoxantrone is an anthracenedione antineoplastic agent used in the treatment of leukemia, lymphoma, breast and prostate cancer; moreover, such drug can induce cardio toxicity in up to 18% of treated patients which is mainly characterized by the development of left ventricular dysfunction, which is manifested as decreased left ventricular ejection fraction and congestive heart failure. Objective: This study is designed to investigate the impacts of graded doses of zinc sulphate on mitoxantrone-induced cardio toxicity in rats via exploring the role of apoptosis in this toxic effect. Methods: Forty-eight adult rats of both sexes were utilized in this study; the animals were randomly divided into six groups of 8 animals each. Group I: distilled water (negative control). Group II: orally-administered zinc sulfate (15mg/kg/day) Group III: orally-administered zinc sulfate (30mg/kg/day).Group IV: Intraperitoneally injected with a mitoxantrone at a dose (2.5 mg/kg) to reach a total cumulative dose of 7.5 mg/kg on day 20. Group V: Orally-administered zinc sulfate at a dose (15mg/kg/day) with an intraperitoneal injection of mitoxantrone at a dose (2.5 mg/kg) was administered to reach the total cumulative dose of 7.5 mg/kg on day 20.Group VI: Orally-administered zinc sulfate at a dose (30 mg/kg/day) with an intraperitoneal injection of mitoxantrone at a dose (2.5 mg/kg) to reach total the cumulative dose of 7.5 mg/kg on day 20. Forty-eight hrs after the end of treatment duration (i.e. at day 22nd), each animal was euthanized by diethyl ether and ketamine, and then after cervical dislocation, the heart of each animal was excised for homogenate preparation to estimate the activity level of caspase-3 enzyme and to detect DNA fragmentation by the utilization of terminaldeoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay. Results: Oral administration of zinc sulfate [(15mg/kg/day with total cumulative dose (7.5 mg/kg) of mitoxantrone] (Group V) resulted in non-significant (P>0.05) difference in caspase-3 level in the cardiac tissue homogenate compared to the corresponding levels in group of rats intraperitoneally injected with total cumulative dose of 7.5 mg/kg of mitoxantrone (Group IV). In contrast, there were significant reduction (P<0.05) incaspase-3 level in the cardiac tissue homogenate of rats orally-administered zinc sulfate [(30 mg/kg/day) with total cumulative dose (7.5mg/kg) of mitoxantrone] (Group VI) compared to the corresponding levels in group of rats intraperitoneally-injected with total cumulative dose of 7.5mg/kg of mitoxantrone (Group IV). In addition, there was an improvement in the immunohistochemistry of rats' heart in Group VI; where, numbers of the apoptotic cells were reduced compared to Group IV. Conclusion: Zinc sulfate at a dose (30 mg/kg/day) inhibits mitoxantrone-triggered cardiomyocyte apoptosis.

Keywords: *Mitoxantrone, Cardiotoxicity, Zinc sulfate, TUNEL-assay, Caspase-3.*

Introduction

Mitoxantrone (MTXN) is a synthetic derivative of doxorubicin. It is considered as an anthracenedione antineoplastic agent. Mitoxantrone acts by intercalating into helical double-stranded DNA causing cross links and strand breaks, thus blocking both DNA and RNA synthesis; and it issued in the treatment of leukemia, lymphoma, breast and prostate cancer; moreover, MTXN was reported to possess immuno-suppressive activity and was approved for use in relapsing and progressive multiple sclerosis (MS) in USA. Such drug was reported to induce cardiotoxicity in up to 18% of treated patients [1, 2]. Clinically, MTX's cardiotoxicity is mainly characterized by the development of left ventricular dysfunction (LVD) manifested as decreased left ventricular ejection fraction (LVEF) and congestive heart failure [3].

Apoptosis has been recommended to play a main role in the therapeutic effects of anthraquinones-based anticancer drugs (anthracyclines and anthracenedione) on tumor cells. Although the molecular mechanisms of cytostatic and cytotoxic action of such classes of drugs are intensively studied, the role of apoptosis in cell death induced by these classes of drugs is still under discussion [4].

Zinc (Zn), an essential trace mineral, is required for the metabolic activity of 300 of the body's enzymes that involved in the metabolism of proteins, carbohydrates, and fats; moreover, it can be considered as an essential mineral for cell division and the synthesis of DNA and protein; and it has an important role in states of cardiovascular diseases (CVDs); and it may have protective effects in coronary artery disease (CAD) and cardiomyopathy [5]. The aim of the study is to investigate the impacts of graded doses of zinc sulphate on MTXN-induced cardiotoxicityin rats via exploring the role of apoptosis in this toxic effect.

Materials and Methods

Preparation of Drugs Solution

Mitoxantrone (MTXN) vial (20mg/10ml) was diluted with 10ml D.W to obtain 1mg/ml to be intraperitoneally (IP) injected at a dose 2.5 mg/kg [6]. Each capsule of zinc sulfate (220mg) was reconstituted in 22ml of D.W to obtain 10mg/ml to be administered by oral gavage at doses 15mg/kg, and 30mg/kg [7].

Animals

Forty-eight (48) healthy adult Wistar Albino rats of both sexes (24 male and 24 female), three months old, weighing range 150-240 gm were utilized in this study; they were gotten from in the Animal House of College of Science, University of Dhi-Qar. Rats were maintained in the College of Science, Wasit University under normal conditions of temperature, humidity and a 12 h light/dark cycle. Rats were supplied with commercial pellets and tap water throughout the experiment period. The animals had no manifestation of any illness upon examination. They were left for two weeks without interference for acclimatization. The study was accepted by the Graduate Studies and the Scientific Committees of the College of Pharmacy, University of Baghdad.

Experimental Protocol

Healthy rats were randomly allocated into six groups, each containing 8 rats' (4 males and 4 females) as follows:

Group I: Rats received 0.5 ml/day of distilled water (DW) intraperitoneally for 20 days. This group served as a negative control.

Group II: Rats orally-administered zinc sulfate at a dose of (15mg/kg/day) alone for 20 days by gavage tube.

Group III: Rats orally-administered zinc sulfate at a dose of (30mg/kg/day) alone for 20 days by gavage tube.

Group IV: Rats intraperitoneally (IP) injected with a MTXN at a dose (2.5 mg/kg) on day 0, 10, 20 to reach total cumulative dose of 7.5 mg/kg on day 20

Group V- Rats orally-administered zinc sulfate at a dose (15mg/kg/day) for 20 days, with an IP injection of MTXN at a dose (2.5 mg/kg) was administered at the day 0, 10, 20 to reach total cumulative dose of 7.5 mg/kg on day 20.

Group VI- Rats orally-administered zinc sulfate at a dose (30 mg/kg/day) for 20 days with an IP injection of MTXN at a dose (2.5 mg/kg) was administered at the day 0, 10, 20

to reach total cumulative dose of 7.5 mg/kg on day 20. Cardiac tissues were obtained to prepare homogenates for the determination of caspase-3 based on ELIZA; where, its levels in the heart tissue homogenate were expressed asng /mL; furthermore, part of the heart were utilized for immunohistochemistry examination by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay.

Estimation of Caspase-3 (CASP-3) in Heart Tissue Homogenate

Rats' heart tissue homogenate was used for the estimation of CASP-3 enzyme activity by using an ELISA kit (Elabscience, USA) [8].

Immunohistochemistry Examination

Myocardial cells apoptosis was investigated by the terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay, which detects fragmentation of DNA in the nucleus during apoptotic cell death *in situ*, was employed using an apoptosis detection kit (Novus, Biological, USA) [9].

Statistical Analysis

Statistical analysis was done by Statistical Package for Social Sciences (SPSS) version 24. Results were expressed as mean ± standard error of means (SEM). Comparison among groups was done by using a one-way Analysis of Variance (One way-ANOVA). The statistically significant differences were considered when $P<0.05$.

Results

Effects on Caspase-3 level

Table 1 and Figure 1 showed that there were non-significant differences *(P*>0.05) in the activity of the caspase-3 enzyme in heart tissue homogenate in group of rats orally-administered zinc sulfate (15 mg/kg/day alone for 20 days) (Group II) compared to negative control rats (Group I). Mean± SEM of caspase-3levels in heart tissue homogenate was respectively, 0.644±0.046 vs. 0.643±0.038.

Similarly, there were non-significant differences (*P*>0.05) in the activity of the caspase-3 enzyme in heart tissue homogenate in the group of rats orally-administered zinc sulfate (30 mg/kg/day alone for 20 days) (Group III) compared to negative control rats (Group I). Mean± SEM in the activity of the caspase-3 enzyme in heart tissue homogenate levels were respectively, 0.653±0.070 and 0.643±0.038. Furthermore, rats IP injected with a total cumulative dose of 7.5 mg/kg of MTXN (Group IV) produced significant elevation (*P*<0.05) in caspase-3levels in heart tissue homogenate compared to the corresponding levels in negative control rats (Group I). Mean± SEM in the activity of caspase-3 enzyme activity levels in heart tissue homogenate were respectively, 1.344±0.086 and 0.643±0.038.

Table 1 and Figure 1. Table 1 and figure 1 also showed that oral administration of zinc sulfate [(15mg/kg/day) with total cumulative dose (7.5 mg/kg) of MTXN] (Group V), resulted in a non-significant (*P*>0.05) difference in the activity of the caspase-3 enzyme levels in heart tissue homogenate compared to the corresponding levels in group of rats IP injected with total cumulative dose of 7.5 mg/kg of MTXN (Group IV).

Mean± SEM of the caspase-3 enzyme levels in heart tissue homogenate were respectively, 0.940±0.117 and 1.344±0.086. Moreover, there were significant reduction (*P*<0.05) in the activity levels of caspase-3 enzyme in heart tissue homogenate of rats orally-administered zinc sulfate [(30 mg/kg/day) with total cumulative dose (7.5 mg/kg) of MTXN] (Group VI) compared to the corresponding levels in rats IP injected with total cumulative dose of 7.5 mg/kg of MTXN (Group IV). Mean± SEM of caspase-3 activity enzyme level in heart tissue homogenate was respectively, 1.344±0.086 vs. 0.792±0.03 Table 1 and figure 1.

In addition, table 1 and figure 1 showed that there were also non-significant differences *(P*>0.05) in the activity levels of the caspase-3 enzyme in heart tissue homogenate of rats orally-administered zinc sulfate [(30mg/kg/day) with total cumulative dose (7.5mg/kg) of MTXN] (Group VI) compared to the corresponding activity levels in heart tissue homogenate of rats orally-administered zinc sulfate [(15mg/kg/day with a total cumulative dose (7.5mg/kg) of MTXN] (Group V). Mean± SEM of the activity levels of the caspase-3 enzyme in heart tissue homogenate levels respectively, 0.792±0.03 vs. 0.940 ± 0.117.

Table 1: Effects of various treatments on caspase-3 level in heart tissue homogenate in rats' groups, No=8

Group	Heart Homogenate Caspase-3 (ng/mL) Mean ± SEM
Group I/ Negative control [Distilled water (DW)]	0.643±0.038
Group II/Zinc sulfate (15 mg/kg/day)	0.644±0.046
Group III/Zinc sulfate (30 mg/kg/day)	0.653±0.070
Group IV/Mitoxantrone (MTXN) (7.5 mg/kg) (total cumulative dose)	1.344±0.086*
Group V/Zinc sulfate (15 mg/kg/day) with mitoxantrone (MTXN) (7.5 mg/kg)	0.940±0.117
Group VI/Zinc sulfate (30 mg/kg/day) with mitoxantrone (MTXN) (7.5 mg/kg)	0.792±0.03#

Data expressed as mean ± standard error of means (SEM)
*: *P*<0.05: Significant difference compared to negative control rats (Group I)
- # *P*<0.05: Significant difference compared to Group IV
N=Number of rats in each group

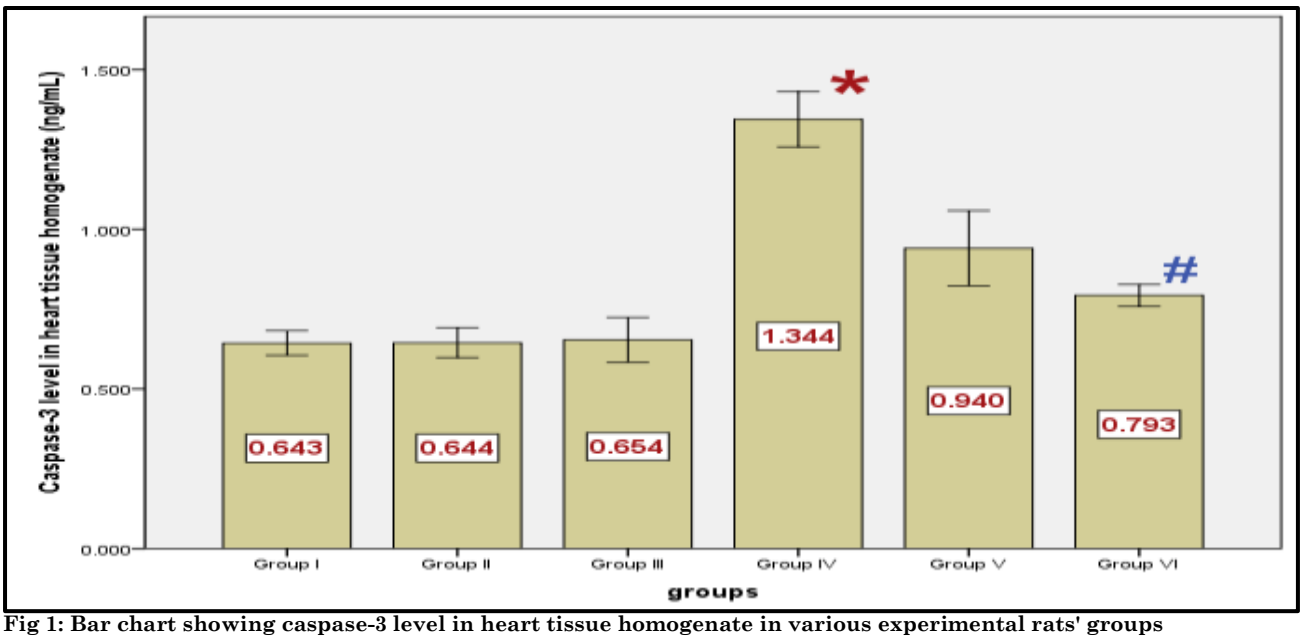

Fig 1: Bar chart showing caspase-3 level in heart tissue homogenate in various experimental rats' groups

Group I: Negative control [Distilled water (DW)]; **Group II:** Zinc sulfate (15mg/kg/day); **Group III:** Zinc sulfate (30mg/kg/day); **Group IV:** Mitoxantrone (MTXN) (7.5mg/kg); **Group V:** Zinc sulfate (15mg/kg/day) with Mitoxantrone (MTXN) (7.5 mg/kg); **Group VI:** Zinc sulfate (30 mg/kg/day) with Mitoxantrone (MTXN) (7.5mg/kg).
-*: Significantly different (*P*<0.05) with respect to the negative control group. **(Group I)**
- # *P*<0.05: Significant difference compared to **Group IV.**

Immunohistochemistry (TUNEL assay) of Rats' Heart Tissue

In sections of heart tissues of -**Group I** (negative control rats administered DW), -**Group II** (rats orally-administered zinc sulfate at a dose of 15 mg/kg/day), and -**Group III** (rats orally-administered zinc sulfate at a dose of 30 mg/kg/day)each for twenty consecutive days showed normal myocardial cells fiber [(no apoptosis (green-coloured cells)] were observed; Figures 2-A, Figure 2-B, and figure 2-C, respectively. Immunohistochemistry changes in the cardiac muscle of rats IP injected with dose of a MTXN at a dose (2.5 mg/kg) on day 0, 10, 20 to reach total cumulative dose of 7.5 mg/kg on day 20 (**Group IV**) were observed that characterized by the presence of apoptotic cells (brown-colored cells) with less number of normal green color of myocardial cells. Figure 2-D. Sections of cardiac muscle of rats orally-administered zinc sulfate at a dose of 15 mg/kg/day for 20 constitutive days with MTXNIP injected at a dose (2.5 mg/kg) on day 0, 10, 20 to reach a total cumulative dose of 7.5 mg/kg on day 20 (**Group V**), figure 2-E showed that apoptotic cells (brown-colored cells) the with less number of normal green color of myocardial cells were observed.

Besides, cardiac muscle section of rats orally-administered zinc sulfate at a dose of 30 mg/kg/day for 20 constitutive days with MTXN IP injected at a dose (2.5 mg/kg) on day 0, 10, 20 to reach total cumulative dose of 7.5 mg/kg on day 20 (**Group VI**) revealed that there were low number of apoptotic cells (brown-coloured cells) and increased number of normal myocardial cells (green-coloured cells). Figure 2-F.

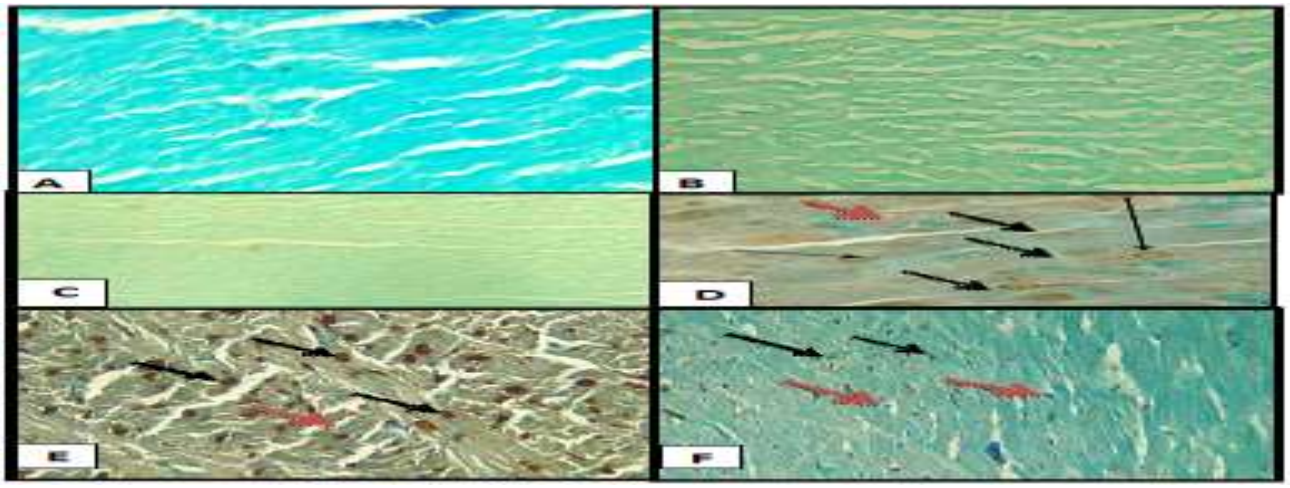

Fig. 2: Immunohistochemistry section of in various experimental rats' groups; (TUNEL assay; ×40). (A) (B) Group II); (C) Group III); (D) Group IV); (E) Group V; (F) Group VI. Normal had green color referred by red arrow and apoptotic cells had brown color referred by black arrow

Discussion

Authors reported that apoptosis can play a substantial role in the therapeutic effects of anthraquinones-based anticancer drugs (anthracyclines and anthracenedione) on tumor cells [10]. It has been suggested that the cytotoxic effect of anthraquinones-based anticancer drugs (doxorubicin as anthracycline drug) is directly connected to the extent of apoptosis they prompt in tumor cells [11].

Additionally, authors reported that administration of MTXN caused apoptosis in cardiac and cancer cells lines and in the human prostate cancer cells [12, 13].Caspases are the main components in the mechanisms responsible for apoptosis, which can be triggered by exogenous stimuli such as inflammation, hypoxia, radiation, and chemotherapeutic drugs; moreover, caspases have a regulated molecular property by removing undesirable cells through controlling auto-digestion process in the organism [14].

Furthermore, CASP-3 was regarded as one marker of apoptosis in the heart tissue; such enzyme is a cytosolic protein that conventionally exists as an inactive precursor with a higher molecular weight (about 32 kDa). When a cell undergoes apoptosis, it cleaved proteolytically into lower molecular weights (11, 17, and 20 kDa) [15]. It has been reported that CASP-3 activation is related to MTXN administration, thus; apoptosis may play a role in the development cardiomyopathy and heart failure via a loss of cardiomyocytes. Therefore, the possibility of cell death by apoptosis was assessed in downstream effect or caspase-3 [16]. In this study, IP injection of total cumulative dose of 7.5 mg/kg MTXN on day 20 (**group IV**) caused a significant elevation in caspase-3 activity (P<0.05) compared to the corresponding activity levels in negative control rats (**group I**),the results of this study are in line with the results of Koceva-Chyła A, *et al*. (2005) [17].

Furthermore, Anghel C *etal*(2015)reported that apoptosis induced by MTXN occurred via the up regulation of the Bax/Bcl-2 ratio (reduced the expression of anti-apoptotic Bcl-2 and increased the expression of pro-apoptotic Bax) and caspase-3 expression, and eventually led to intermediate filaments disruption and cell death but without compromising its antitumor benefits [18].

The current study showed that orally-administered zinc sulfate (15mg/kg/day) **(Group II)** and orally-administered zinc sulfate (30mg/kg/day) **(Group III**) each alone produced a non-significant differences in heart tissue homogenate CASP-3activity level (P>0.05) compared to negative control rats (table 1). Besides, orally-administered zinc sulfate at a dose (15mg/kg/day) with an IP injection of MTXN at a total cumulative dose of 7.5 mg/kg on day 20 **(Group V**) produced non-significant differences (P>0.05) in heart tissue homogenate CASP-3compared to MTXN-treated rats(**Group IV**) [table 1 and figure 1]; additionally, orally-administered zinc sulfate at a dose

(30mg/kg/day) with an IP injection of MTXN at total cumulative dose of 7.5 mg/kg on day20 resulted in significant reduction (P<0.05) in the activity level of CASP-3 compared to the corresponding activity levels in MTXN-treated rats (**Group IV**) [table 1 and figure 1]; this may predict the anti-apoptotic effect of the higher dose of zinc sulfate, and may have a protective effect on cardiomyocytes.

Results of the current study are in tune with those of others concerning the impact of zinc on CASP-3 level in the heart tissue homogenate [7] but by the utilization of diabetic models. Eron SJ *et al* at 2018 mentioned that, deficiency of zinc increased apoptosis in adult animals; furthermore, zinc has shown to have a protective effect by targeting apoptotic proteins, particularly the caspases [19].

Moreover, Velazquez-Delgado EM and Hardy JA (2012) reported that zinc hadanti-apoptotic function by blocking caspase activity; also authors mentioned that Znmay have a high affinity for the amino acids His^{237} and Cys^{285} present on the enzyme, and this interaction may be responsible for its inhibitory effect; furthermore, it has also been suggested that zinc may prevent the processing of CASP-3 with its upstream initiator enzyme, caspase-9, but not by blocking the even earlier step of cytochrome *c* release from the mitochondria [20].

In addition, zinc may act as an inhibitor of the endonuclease that is responsible for apoptotic DNA degradation; moreover, it has been stated that zinc considered as a potent inhibitor of the protease CASP-3 [21, 22].

Furthermore, immunohistochemical changes observed in the current study that detect DNA fragmentation and apoptosis through terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining procedure of myocardial cells in MTXN-treated rats**(group IV**) is characterized by increased apoptotic cells, which have brown color as shown in figure 2-Dcompared to negative control rats**(group I)** (figure 2-A); where, normal myocardial cells represented by green color were observed; these results were in agreement with those of Anghel C, *et al* at 2015 [18] who found that MTXN caused apoptosis by increased numbers of TUNEL positive nuclei in mouse heart.

In addition a study of See-Hyoung Park and his colleague at 2018 reported that MTXN caused apoptosis in osteosarcoma cells by the utilization of TUNEL assay [23].Rats orally administered zinc sulfate at a dose of 15 mg/kg/day (**Group II**) and 30 mg/kg/day (**Group III**) each alone for twenty consecutive days showed normal myocardial cells fiber (not apoptotic, green-coloured cells) as shown in figures 2-B, figure 2-C, respectively.

Also in this study, the immunohistochemical examination of TUNEL-staining in groups of rats treated zinc sulfate at a dose of 15 mg/kg/day with MTXN (**Group V**) showed that the presence of apoptotic cells (brown-colored cells) with less number of normal green color of myocardial cells (figures 2-E; while, in heart sections of rats orally-administered zinc sulfate at a dose of 30 mg/kg/day with MTXN (**Group VI**) there were reduction in apoptotic cells (brown color) with an increased number of normal myocardial cells (green color); Figure 3-F] compared to those observed in **Group IV** (MTXN-treated).

These results are in consistent with those reported by Korkmaz-Icöz S *et al* who showed that treatment of diabetic rats with $Zn(ASA)_2$, the number of cardiac apoptotic nuclei was significantly decreased [24]. Similarly, results of the present study are consistent with those reported by *John P. M. et al*[25] who indicated that low concentration of zinc sulfate had no effect on cultured human retinal pigment epithelial cells but the high concentration of zinc had a protective effect on such cells by reducing the amount of TUNEL-positive nuclei. Moreover, Fadi Choucaire *et. al.* reported that zinc caused significant reduction in sperm DNA fragmentation [26].

Furthermore, Susmita Barman and Krishnapura Srinivasan reported that Zn supplementation had protective effect against apoptosis partially by restoring the balance of Bax and Bcl-2 proteins; where, suppression of the elevated level of Bax and up-regulation of Bcl-2 expression suggested the anti-apoptotic effect of Zn [27]. To our knowledge, the current study is the first that investigate the impact of zinc sulfate on caspase-3 enzyme level and myocardial apoptosis in MTXN-induced cardiotoxicity in rats, thus we could not have a chance to

compare results with others concerning this respect.

Conclusion

From results obtained from this study, it could be concluded that zinc sulfate at a dose (30 mg/kg/day) inhibits mitoxantrone-triggered cardiomyocyte apoptosis.

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