



شبكة المعلومات الجامعية
التوثيق الإلكتروني والميكروفيلم

بسم الله الرحمن الرحيم



MONA MAGHRABY



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التوثيق الإلكتروني والميكروفيلم

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MONA MAGHRABY



Studying modulation of calcium in cardiotoxicity induced by doxorubicin using vitamin D and vitamin B₃

Thesis presented by

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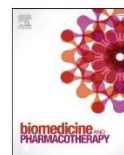
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Comparative study on beneficial effects of vitamins B and D in attenuating doxorubicin induced cardiotoxicity in rats: Emphasis on calcium homeostasis

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ABSTRACT

The use of doxorubicin (DOX) to treat various tumors is limited by its cardiotoxicity. This study aimed to investigate and compare the cardioprotective effects of nicotinamide (NAM) and alfacalcidol ($1\alpha(\text{OH})\text{D}_3$), against DOX-induced cardiotoxicity. Sprague Dawley male rats received DOX (5 mg/kg, i.p.) once/week for four consecutive weeks. Treated groups received either NAM (600 mg/kg, p.o.) for 28 consecutive days or $1\alpha(\text{OH})\text{D}_3$ (0.5 ug/kg, i.p.) once/week for four consecutive weeks. DOX elicited marked cardiac tissue injury manifested by elevated serum cardiotoxicity indices, conduction and histopathological abnormalities. Both NAM and $1\alpha(\text{OH})\text{D}_3$ successfully reversed all these changes. From the mechanistic point of view, DOX provoked intense cytosolic and mitochondrial calcium (Ca^{2+}) overload hence switching on calpain1 (CPN1) and mitochondrial-mediated apoptotic cascades as confirmed by upregulating Bax and caspase-3 while downregulating Bel-2 expression. DOX also disrupted cardiac bioenergetics as evidenced by adenosine triphosphate (ATP) depletion and a declined ATP/ADP ratio. Moreover, DOX upregulated the Ca^{2+} sensor; calmodulin kinase II gamma (CaMKII- γ) which further contributed to cardiac damage. Interestingly, co-treatment with either NAM or $1\alpha(\text{OH})\text{D}_3$ reversed all DOX associated abnormalities by preserving Ca^{2+} homeostasis, replenishing ATP stores and obstructing apoptotic events. Additionally, DOX prompted nuclear factor kappa B (NF- κB) dependent inflammatory responses and subsequently upregulated interleukin-6 (IL-6) expression. Co-treatment with NAM or $1\alpha(\text{OH})\text{D}_3$ effectively obstructed these inflammatory signals. Remarkably, NAM showed superior beneficial cardioprotective properties over $1\alpha(\text{OH})\text{D}_3$. Both NAM and $1\alpha(\text{OH})\text{D}_3$ efficiently attenuated DOX-cardiomyopathy mainly via preserving Ca^{2+} homeostasis and diminishing apoptotic and inflammatory pathways. NAM definitely exhibited effective cardioprotective capabilities over $1\alpha(\text{OH})\text{D}_3$.

1. Introduction

Doxorubicin (DOX), an anthracycline antibiotic, is considered the mainstay in the treatment of a wide variety of human malignancies such

as breast, lung and ovarian cancers [1], notwithstanding its potent antitumor effect, the use of DOX has been obstructed by several deleterious and fatal adverse effects hindering its clinical use [2]. Cardiotoxicity is considered the most significant and frequent toxicity

Abbreviations: ACASP-3, activated caspase-3; ATP, Adenosine triphosphate; ADP, Adenosine diphosphate; $1\alpha\text{OHD}_3$, alfacalcidol; BW, Body Weight; Ca^{2+} , Calcium; CPN-1, Calpain-1; CaMKII δ , Calcium calmodulin Kinase II δ ; Ctn1, Cardiac Triponin-1; CK-MB, Creatine kinase isoenzyme-MB; DOX, Doxorubicin; ECG, Electrocardiography; ELISA, Enzyme-linked immunosorbent assay; EM, Electron Microscope; HW, Heart Weight; IL-6, interleukin-6; LDH, Lactate dehydrogenase; i.p., intraperitoneal; NAD⁺, nicotinamide adenine dinucleotide; NAM, Nicotinamide; NF κ B, Nuclear Factor Kappa B; PBS, phosphate buffer saline; RyR2, Ryanodine receptor; SERCA2a, Sarcoplasmic/ endoplasmic reticulum Ca^{2+} -ATPase 2a; SR, Sarcoplasmic Reticulum.

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associated with DOX [3]. A multitude of mechanisms have been implicated in the pathogenesis of DOX-induced cardiotoxicity. One of the profound mechanisms associated with DOX cardiotoxicity is linked to Ca^{2+} homeostasis dysregulation in cardiac tissue [4]. DOX down-regulates the expression of sarcoplasmic/endoplasmic reticulum Ca^{2+} ATPase2a (SERCA2a), a Ca^{2+} pump responsible for Ca^{2+} influx from cytosol to sarcoplasmic reticulum. Consequently, DOX elevates both cytosolic and mitochondrial Ca^{2+} levels [5]. This cytosolic Ca^{2+} level elevation is responsible for Ca^{2+} /calmodulin-dependent protein kinase II-gamma (CaMKII γ) activation which contributes considerably towards the pathogenesis of DOX cardiotoxicity [6]. Activated CaMKII γ promotes apoptosis through the calcium-dependent, non-lysosomal cysteine proteases enzyme calpain1 (CPN1) [7]. Moreover, whenever a certain threshold of mitochondrial Ca^{2+} level is reached, the opening of a mitochondrial permeability transition pore is triggered, allowing the release of cytochrome-C into the cytosol and hence formation of apoptosome, which in turn activates caspase-9 and ultimately result in apoptotic cell death [8]. DOX also prompts inflammatory responses via activation of the transcription of the nuclear factor kappa B (NF- κ B) and consequent release of pro-inflammatory cytokines in the myocardium [9].

Recently, a diversity of natural compounds were investigated for their potential competences in mitigating DOX-induced cardiotoxicity [10]. Vitamins are among the biologically efficient compounds that have been reported extensively to alleviate cardiovascular disorders [11]. Nicotinamide (NAM) is the amide form of vitamin B₃ [12]. Vitamin B₃ have two common functional co-factors which are nicotinamide adenine dinucleotide (NAD⁺), nicotinamide adenine dinucleotide phosphate (NADP⁺) and their reduced forms (NAD(P)H). These cofactors, referred to as the NAD(P)H pool, are intimately involved in all essential bioenergetics, anabolic and catabolic pathways [13]. This pool also has crucial roles in cell metabolism and cell signaling through multiple pathways regulating intracellular Ca^{2+} signaling, mitochondrial respiration and ATP production. Manipulation of NAD⁺ bioavailability through vitamin B₃ supplementation has become a valuable nutritional and therapeutic avenue [14].

Vitamin D (1,25(OH)₂D₃) is a multifunctional micronutrient that is vital for human health [15]. Growing evidence suggests that low serum 1,25(OH)₂D₃ levels are associated with high risk of cardiovascular disease such as hypertension, coronary artery disease, ischemic heart disease and strokes [16]. Indeed, endogenous 1,25(OH)₂D₃ is important in inhibiting inflammatory pathways and modulating proinflammatory cytokines production protecting tissues from hyperinflammatory response damage [17,18]. Furthermore, 1,25(OH)₂D₃ is a potent anti-oxidant facilitating the balance of mitochondrial activities and preventing oxidative stress-related protein oxidation, lipid peroxidation, and DNA damage [15]. Previous studies reported that hypovitaminosis D is associated with an elevation of intracellular Ca^{2+} and acceleration in cellular damage, apoptosis, and aging [15].

Vitamins as nutraceuticals offer a safe and bright prospect for future treatment and the prevention of cardiovascular toxicities and diseases. Accordingly, the aim of this study was to compare the potential protective effects of NAM and 1 α (OH)D₃ in DOX-induced chronic cardiotoxicity through the elucidation of intracellular Ca^{2+} signaling that can greatly modulate inflammation, and the apoptotic status of the cardiomyocytes.

2. Material and methods

2.1. Material

Doxorubicin (DOX) was purchased as Adriablastine (50 mg DOX hydrochloride, Pharmacia and Upjohn, Milan, Italy). NAM was purchased from Sigma Chemical Co. (St.Louis, MO, USA). Vitamin D was purchased as One Alpha IV injection (alfacalcidol) from LEO Pharma (Hurley, Berkshire, UK). Formaldehyde 37% and phosphate buffered

glutaraldehyde were purchased from El-Nasr Chemical Co. (Egypt). All other chemicals were of the highest commercially available grade of purity.

2.2. Animals

Adult Sprague Dawley male rats weighing between 150 and 220 gm (age: 10–12 weeks) were obtained from Nile Co. for Pharmaceutical and Chemical industries (Cairo, Egypt). The animals were acclimatized for two weeks before experimentation in an air-conditioned atmosphere at a temperature of 25 °C with alternating 12-hour light and dark cycles and allowed free access to standard diet pellets and water. The experimental protocol was conducted according to the ethical guidelines and was approved by the Research Ethics Committee of the Faculty of Pharmacy, Ain Shams University, Egypt under the Memorandum No. 54.

2.3. Experimental design

Based on our preliminary study (supplementary), the optimal cardioprotective dose of NAM was 600 mg/kg and 1 α (OH)D₃ (0.5 ug/kg). The duration of the experiment was 28 days. Sixty adult male Sprague Dawley rats (150–220 g) were weighed and assigned randomly into six groups (10 rats per group). **The control group:** received distilled water through an oral gavage daily for 28 days. **The DOX group:** was given a single intraperitoneal (i.p.) injection of DOX (5 mg/kg) once a week for four consecutive weeks [19]. **The NAM + DOX group:** received an oral dose of NAM (600 mg/kg) daily for 28 days and DOX (once weekly, 5 mg/kg). **The 1 α (OH)D₃ + DOX group:** received i.p. injection of 1 α (OH)D₃ (0.5 ug/kg) [20] once weekly 48 h before the DOX injection (once weekly of 5 mg/kg) for four consecutive weeks. **The NAM group:** received NAM at an oral dose of 600 mg/kg daily for four consecutive weeks. **The 1 α (OH)D₃ group:** received 1 α (OH)D₃ (0.5 ug/kg) i.p. once weekly for four consecutive weeks (Fig. 1).

Forty-eight hours after the last DOX injection, the animals were weighed, anesthetized with ketamine (75 mg/kg; i.p.) and then subjected to ECG recording (Fig. 1). Blood samples were withdrawn from the retro-orbital plexus and allowed to clot. The blood samples were then centrifuged, and the serum was collected for biochemical analysis. Heart weights (HW) were recorded. Heart tissues were collected and homogenized in phosphate buffer saline (PBS). The homogenate was subsequently used for biochemical analysis. Some heart specimens were fixed in 10% buffered formalin for histopathological and immunohistochemical examination and other heart specimens were fixed immediately in 2.5% phosphate buffered glutaraldehyde (pH 7.4) for electron microscopic (EM) examination.

2.4. Electrocardiography (ECG)

The rats were anesthetized with ketamine (75 mg/kg; i.p.) and an electrocardiography (ECG) was recorded for 1 min. Heart rate, P duration, QRS Interval and QTc, were monitored using ECG Power Lab module which consists of Power-Lab/8sp and Animal Bio-Amplifier, Australia, in addition to Lab Chart7 software with ECG analyzer.

2.5. Determination of body and heart weights

The initial and final body weight (BW) of the rats and their HW were recorded for all study groups and their heart indices were calculated using the formula (HW / BW) \times 100.

2.6. Assessment of serum cardiotoxicity indices

Serum creatine kinase isoenzyme-MB (CK-MB) and serum lactate dehydrogenase (LDH) activities were determined spectrophotometrically following the standard procedures using commercial kits obtained from Spectrum diagnostics, Cairo, Egypt. The serum level of cardiac

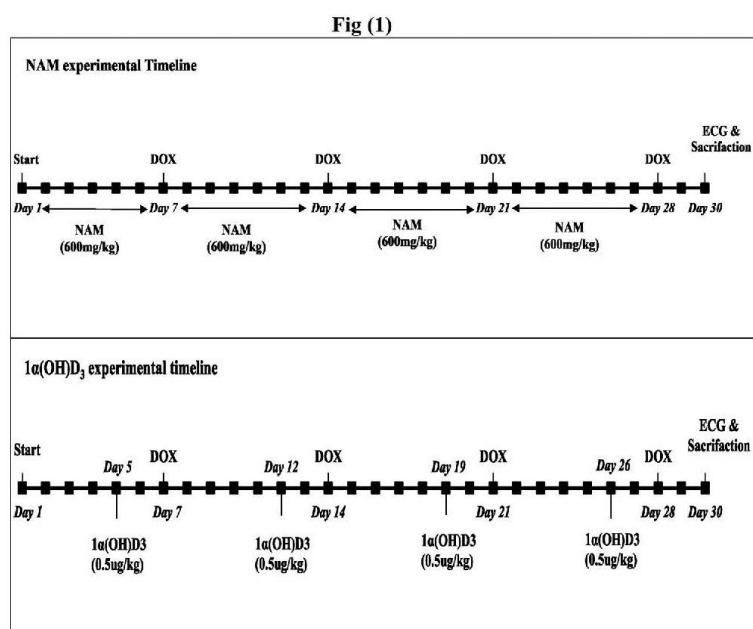


Fig. 1. Timeline of experimental study design and drug administration.

troponin I (c-TnI) was determined using enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions developed by Life Diagnostics Inc.

2.7. Histopathological and electron microscopic examination

Heart samples were processed for light and electron microscopy. For light microscopy, heart specimens were prepared and stained with hematoxylin and eosin (H and E). They were fixed in 10% formalin for 24 h and then washed with tap water, before using serial dilutions of alcohol for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56°C in hot water oven for 24 h. Paraffin bees wax tissue blocks were prepared for sectioning at 4 µm thickness by the slide microtome. Heart specimens were immediately fixed in 2.5% phosphate buffered glutaraldehyde (pH 7.4) at 4 °C for 24 h and post fixed in 1% osmium tetroxide for 1 h, then dehydrated in ascending grades of ethanol for electron microscopic examination [21].

2.8. Assessment of cell viability markers

Heart tissues were homogenized in ice-cold PBS (0.02 mol/L, pH 7.0–7.2). The resulting homogenates were centrifuged for 15 min at 5000 rpm. The supernatant was separated, and assay was carried out following the standard procedures using the competitive inhibition enzyme immunoassay technique according to the manufacturer's instructions developed by My BioSource, Inc. for assessing both ATP and ADP content.

2.9. Assessment of cytoplasmic and mitochondrial calcium (Ca^{2+})

The mitochondria were isolated using the differential centrifugation method [22]. Heart specimen homogenate was centrifuged for 10 min at 600 rpm. The supernatant was centrifuged at 15,000 rpm for 5 min. The supernatant was the cytosolic fraction. The mitochondrial fraction was

obtained from the pellet, after washing it with a buffer (containing sucrose 0.25 M, Tris-HCl 5 mM, potassium dihydrogen phosphate 3 mM and magnesium chloride 5 mM) and centrifuging it at 15,000 rpm for 5 min. The last step was repeated twice to ensure pure mitochondrial fraction. The spectrophotometric technique was used to determine the measurements of both cytosolic and mitochondrial Ca^{2+} levels [23]. Briefly, 25 µL of sample was incubated at room temperature with 500 µL of color reagent and 500 µL 2-amino-2-methylpropanol buffer for 15 min and then measured at 540 nm.

2.10. Assessment of calcium calmodulin kinase II gamma (CaMKII δ)

The concentration of CaMKII- δ was assessed in the tissue homogenates using ELISA kits according to the manufacturer's instructions developed by Cloud-Clone Corp. USA.

2.11. Assessment of calpain1 (CPN1)

The calpain1 (CPN1) concentration was assessed in the tissue homogenates using ELISA kits according to the manufacturer's instructions (Bioassay Technology, China).

2.12. Assessment of apoptotic markers

Immunohistochemical staining was performed according to the manufacturer's protocol as previously described. Deparaffinized tissue sections were incubated with one of the following primary antibodies: mouse monoclonal BAX primary antibody (Thermo Fisher Scientific, Cat.#MA5-14003), rabbit polyclonal primary antibody Bcl-2 (Thermo Fisher Scientific, Cat.#PA1-30411), rabbit polyclonal primary antibody caspase-3 (Thermo Fisher Scientific, Cat.#RB-1197-R7). Quantitative measurement of activated caspase-3 (ACASP-3) in tissue homogenate was assessed using ELISA kits according to manufacturer's instructions developed by Cloud-Clone Corp. USA (SEA396Mu).

2.13. Assessment of inflammatory markers

ELISA was used for assessing the concentration of the inflammatory marker IL-6 in tissue homogenates according to the manufacturer's instructions (Bioassay Technology, China). Quantitative determination of nuclear NF- κ B concentration was performed according to the manufacturer's protocol (Elabsience Biotechnology, Cat.#E-EL-R0917). Meanwhile immunohistochemistry was performed to determine of NF- κ B expression in cardiac tissues. Immunohistochemical staining was performed according to the manufacturer's protocol. Deparaffinized tissue sections were incubated with the primary antibody: rabbit polyclonal NF- κ B antibody (Thermo Fisher Scientific, Cat. #RB-1638-P0).

For all immunohistochemical staining of BAX, Bcl-2, caspase-3 and NF- κ B, four non-overlapping fields (magnification, $\times 400$) were randomly selected per tissue section of each sample to determine the positive immune-expression percentage levels. Morphological measurements and analyzed data were obtained using the Leica application module for tissue sections analysis attached to a Full HD microscope imaging system (Leica Microsystems GmbH, Germany). Image quantitation was performed using image software (version 1.48) to calculate the area percent (A%).

2.14. Statistical analysis

Data were presented as mean \pm S.D. Statistical analysis was performed using one-way analysis variance (ANOVA) followed by Tukey-Kramer Post Hoc Test. The 0.05 level of probability was used as the criterion for significance. All statistical analyses were performed using Instat software package (version 8.4.2). Graphs were sketched using GraphPad prism software version 8 (ISI® software, USA).

3. Results

3.1. Effect of NAM or 1 α (OH) D_3 on abnormal ECG

DOX intoxication provoked a significant bradycardia, and prolonged QRS complex, PR interval and QTc interval durations, compared with the control group. In contrast, compared with the DOX intoxicated group, co-administration of either NAM or 1 α (OH) D_3 effectively reversed all the DOX-induced ECG abnormalities as evidenced by elevating the heart rate and decreasing the duration of both the QRS complex, PR interval and QTc interval (Fig. 2). However, no significant differences were detected between NAM or 1 α (OH) D_3 + DOX intoxicated groups.

3.2. Effect of NAM or 1 α (OH) D_3 on the final body weights and cardiac weight indices

DOX-treated rats showed a significant reduction in final BW by 18% and a significant increase in cardiac weight and cardiac index by 19% and 46%, respectively compared with the control group. On the other hand, NAM co-treatment of intoxicated rats significantly increased their BW by 11.93% and reduced both their cardiac weight and index by 13% and 22%, respectively compared with the DOX group. Rats co-treated with 1 α (OH) D_3 and DOX showed a significant increase in BW by 6.89% and a significant decrease in cardiac weight and index by 12% and 19%, respectively compared with the DOX group. In comparison to 1 α (OH) D_3 co-treatment, the NAM administration showed a significant reduction in cardiac index by 5% (Table 1).

3.3. Effect of NAM or 1 α (OH) D_3 on the serum cardiotoxicity indices

As markers for myocardial injury, the activities of CK-MB and LDH enzymes and expression of c-TnI were assessed in serum. Serum CK-MB, LDH and c-TnI were significantly elevated in the DOX group by 31%, 12% and 25%, respectively, when compared with the control group.

NAM co-treatment showed a significant reduction in serum CK-MB, LDH and c-TnI levels by 16%, 11% and 14%, respectively, compared with the DOX group. Meanwhile, 1 α (OH) D_3 co-treatment induced significant reduction in the activities of serum CK-MB, LDH and c-TnI levels by 10%, 6% and 13%, respectively, when compared with the DOX group (Table 2). No significant difference in CK-MB, LDH or c-TnI was detected between the DOX-intoxicated rats co-treated with either NAM or 1 α (OH) D_3 .

3.4. Effect of NAM or 1 α (OH) D_3 on histopathological and electron microscope alterations

Heart tissues from the control group showed a normal appearance and arrangement of cardiac muscle fibers (Fig. 3A). On the contrary, DOX intoxication prompted prominent histopathological changes in cardiac tissues in the form of a disarrangement and degeneration of the myocardial muscle and widespread vacuolization, focal pyknosis in the myocardium associated with congestion in the myocardial blood vessels (Fig. 3B). Interestingly, cotreatment with either NAM or 1 α (OH) D_3 along with DOX efficiently improved these histopathological abnormalities. Indeed, NAM co-treatment exerted more obvious cytoprotective effects on the myocardial cells as shown by intact cardiomyocytes and minimal degenerated and fragmented muscle cells (Fig. 3C) while 1 α (OH) D_3 co-treatment still showed wide areas of degenerative changes of cardiomyocytes with intercellular spaces (Fig. 3D). Treatment with either NAM or 1 α (OH) D_3 alone showed no alterations in the normal cardiac myocyte architecture (Fig. 3E and F).

Upon EM examination, the transmission electron micrographs of the control group showed linear muscle fiber composed of several cardiac muscle cells joined end to end at specialized junctional zones. Each cell had an elongated nucleus centrally located in the sarcoplasm that bound by a sarcolemma. The mitochondria were uniform in size. The myofibrils are formed of regular sarcomeres with distinct Z lines (Fig. 4A). Meanwhile, the DOX treated group showed disorganized cardiomyocytes with irregular shaped nuclei characterized by an aggregation of nuclear chromatin and dissociation of the outer nuclear membrane and perinuclear edema. The mitochondria showed significant disarrangement and non-uniform size. The variation in mitochondrial size is due to the continuous replacement of damaged mitochondria by newly synthesized ones to sustain the constant need for ATPs. The non-uniform sized electron dense mitochondria were characterized by enlarged size, loss of inter-mitochondrial contacts, mitochondrial cavitation, swelling and fragmentation. In addition, the DOX-treated group showed areas of myofibril lysis, disordered arrangement and loss of Z- and M- bands together with a loss of normal sarcomere appearance. Interestingly, some vessels showed damage to the endothelial lining (Fig. 4B and C). Meanwhile, the NAM+DOX group showed myofibrils formed of regular sarcomeres with minimal areas of myofibril lysis and mitochondria of a nearly uniform size (Fig. 4D). On the other hand, the 1 α (OH) D_3 + DOX treated group showed arranged cardiac muscle fibers and cells joined end to end at specialized junctional zones. However, the mitochondria were not uniform in size and arranged irregularly and each cell had an elongated nucleus centrally located in the sarcoplasm (Fig. 4E). Interestingly, the EM examination of the myocardium after administration of NAM showed better reservation of mitochondrial integrity and size compared with 1 α (OH) D_3 . Finally, the muscle fiber in both NAM and 1 α (OH) D_3 groups showed normal architecture features of cardiac cells (Fig. 4F and G).

3.5. Effect of NAM or 1 α (OH) D_3 on cardiomyocytes viability markers

In the DOX group, the ATP/ADP ratio was significantly decreased by 76%, as compared with the control group. When compared with the DOX group, the NAM+DOX treated group presented a significant increase in ATP/ADP ratio by 319%. Meanwhile, rats treated with 1 α (OH) D_3 + DOX exhibited significant elevation ATP/ADP ratio by 230% as

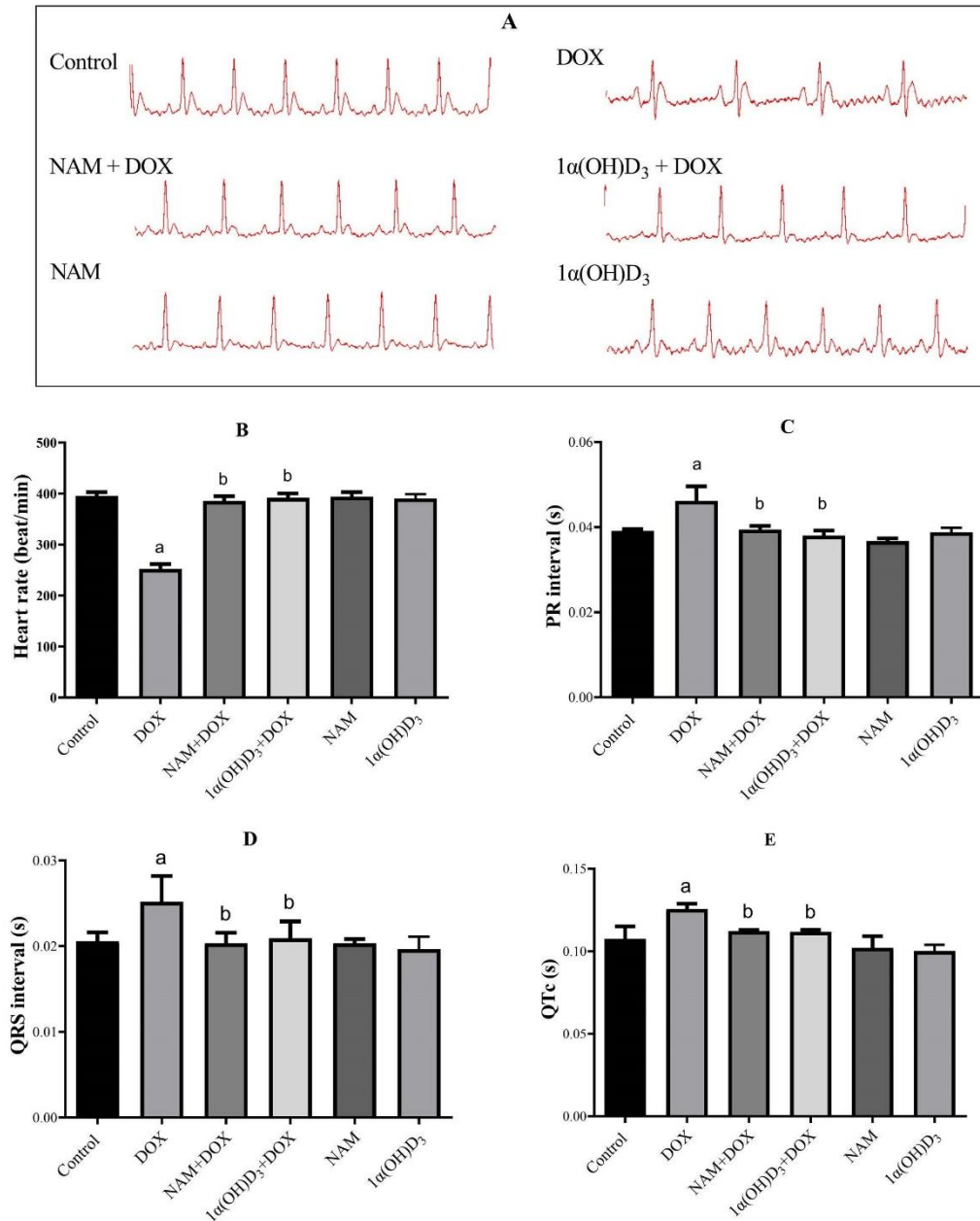


Fig. 2. Effect of NAM or 1α(OH)D₃ on ECG abnormalities in DOX-induced cardiotoxicity. (A) ECG graph, (B) Heart rate (beat/min), (C) PR interval (s), (D) QRS duration (s) (E) QTc interval (s). Data are represented as mean ± SD (n = 8–10). a,b,c: statistically significant from control, DOX or NAM + DOX, respectively at P < 0.05 using one-way ANOVA followed by Tukey-Kramer Post Hoc test.

Table 1
Effect of NAM or 1 α (OH)D₃ on body weight, heart weight, cardiac index and survival%.

Treated groups	Body Weight (gm)		Heart Weight (gm)	Cardiac index x10 ³	Survival%
	Initial	Final			
Control	212.5 ± 7.09	251.1 ± 6.46	0.57 ± 0.02	2.20	100
DOX	217.7 ± 5.61	206.1 ± 9.75 ^a	0.67 ± 0.018 ^a	3.26 ^a	80
NAM + DOX	216.0 ± 5.46	230.7 ± 6.79 ^{a,b}	0.59 ± 0.004 ^{a,b}	2.57 ^{a,b}	100
1 α (OH)D ₃ + DOX	215.8 ± 7.86	220.3 ± 6.22 ^{a,b}	0.59 ± 0.009 ^{a,b}	2.67 ^{a,b,c}	100
NAM	214.7 ± 6.32	247.0 ± 7.76	0.56 ± 0.02	2.28	100
1 α (OH)D ₃	213.7 ± 7.90	250.9 ± 9.33	0.56 ± 0.023	2.22	100

Data are represented as mean ± SD (n = 8–10). a, b and c: Statistically significant from the control, DOX or NAM + DOX group, respectively at P < 0.05 using one-way ANOVA followed by Tukey-Kramer Post Hoc test.

Table 2
Effect of NAM or 1 α (OH)D₃ on serum CK-MB, LDH and cardiac Troponin I (cTnI).

Treated groups	CK-MB (U/L)	LDH (U/L)	cTnI (ng/ml)
Control	31.63 ± 1.85	50.37 ± 1.45	3.62 ± 0.103
DOX	41.37 ± 1.46 ^a	56.55 ± 1.38 ^a	4.54 ± 0.163 ^a
NAM + DOX	34.73 ± 1.89 ^b	51.72 ± 1.35 ^b	3.89 ± 0.103 ^{a,b}
1 α (OH)D ₃ + DOX	33.55 ± 2.14 ^b	52.90 ± 2.53 ^b	3.97 ± 0.154 ^{a,b}
NAM	32.83 ± 1.08	50.35 ± 1.38	3.67 ± 0.114
1 α (OH)D ₃	31.85 ± 1.12	50.37 ± 1.88	3.68 ± 0.111

Data are represented as mean ± SD (n = 8–10). a or b: Statistically significant from the control or DOX group, respectively at P < 0.05 using one-way ANOVA followed by Tukey-Kramer Post Hoc test.

compared with the DOX group. Interestingly, NAM + DOX treated group presented a significant increase in the ATP/ADP ratio by 27% as compared with the 1 α (OH)D₃ + DOX (Fig. 5C).

3.6. Effect of NAM or 1 α (OH)D₃ on cytosolic and mitochondrial Ca²⁺ levels

The cytosolic Ca²⁺ level in DOX treated rats increased significantly by 37% compared with the control group. Meanwhile, co-treatment with either NAM or 1 α (OH)D₃ significantly decreased cytosolic Ca²⁺ levels by 26% and 19%, respectively, as compared with the DOX treated group. Interestingly, NAM + DOX treated group significantly decreased the cytosolic Ca²⁺ level by 8% compared to 1 α (OH)D₃ + DOX group (Fig. 6A). Similar results were obtained concerning the mitochondrial Ca²⁺ level which DOX significantly increased by 447% as compared with the control group. Co-treatment with both NAM and 1 α (OH)D₃ perfectly attenuated the imbalance of mitochondrial Ca²⁺ overload caused by DOX. NAM and 1 α (OH)D₃ significantly decreased the mitochondrial Ca²⁺ level by 80% and 76%, respectively as compared with the DOX treated group. NAM co-treatment was more effective in preserving the mitochondrial Ca²⁺ load where it demonstrated more significant reduction in the mitochondrial Ca²⁺ level by 18% as compared with the 1 α (OH)D₃ + DOX group (Fig. 6B).

3.7. Effect of NAM or 1 α (OH)D₃ on calmodulin Kinase II gamma (CaMKII γ) content

There was a significant increase in CaMKII- γ by 30% in DOX-intoxicated rats as compared with the control group. On the other hand, NAM + DOX and 1 α (OH)D₃ + DOX treated groups showed significant reduction in CaMKII- γ levels by 18% and 12%, respectively, compared with the DOX group. NAM+DOX-treated rats showed a more significant reduction by 7% over 1 α (OH)D₃ + DOX-treated animals (Fig. 7A).

3.8. Effect of NAM or 1 α (OH)D₃ on calpain 1 (CPN1)

There was a significant increase in the CPN1 content induced by DOX

by 206% as compared with the control group. On the other hand, NAM co-treatment showed a significant decline in the CPN1 content by 45% as compared with the DOX-treated group. In parallel, co-treatment with 1 α (OH)D₃ with DOX significantly reduced the CPN1 content by 38% as compared with the DOX group. Remarkably, there was a significant reduction in the NAM+DOX treated group by 12% as compared with 1 α (OH)D₃ + DOX-treated rats (Fig. 7B).

3.9. Effect of NAM or 1 α (OH)D₃ on apoptotic markers expression in myocardial tissues

Apoptotic changes induced by DOX were assessed by an immunohistochemical examination of Bax, Bcl-2 and caspase-3 proteins and the quantitative analysis of ACASP-3 (Figs. 8 and 9). DOX decreased the anti-apoptotic Bcl-2 and increased the pro-apoptotic Bax as compared with the control group. The semiquantitative analysis of the immunostaining showed a significant elevation in Bax: Bcl-2 ratio in DOX-treated rats compared with the control group. Moreover, each of the NAM and 1 α (OH)D₃ co-administration ameliorated the DOX intoxication by increasing expression of Bcl-2 and decreasing BAX expression (Fig. 8B and C). In parallel a reduction in Bax: Bcl-2 ratio as compared with the DOX treated group was observed in both vitamins-cotreated groups (Fig. 8D).

The apoptotic effects of DOX were further confirmed by assessing caspase-3 expression where DOX extensively elevated the enzyme expression as shown by the intense immunostaining as compared with the control group. On the other hand, both NAM and 1 α (OH)D₃ cotreatment markedly reduced caspase-3 expression as evidenced by minimal immunostaining (Fig. 9G). Upon comparison, there was no significant change between NAM or 1 α (OH)D₃ co-treated groups in Bax, Bcl-2 and caspase-3 expression.

The apoptotic effect of DOX was further supported by a 121% increase in ACASP-3 levels as compared with the control group. When compared with the DOX-treated group, NAM co-treated animals showed a 17% significant decline in ACASP-3 content. Meanwhile, 1 α (OH)D₃ co-treated group showed a significant reduction in ACASP-3 by 8% as compared with the DOX group. Remarkably, there was a significant reduction in the NAM+DOX-treated group by 10% as compared with 1 α (OH)D₃ + DOX-treated rats (Fig. 9H).

3.10. Effect of NAM or 1 α (OH)D₃ on inflammatory markers in myocardial tissues

The pro-inflammatory responses induced by DOX were manifested by assessment of the inflammatory markers; NF- κ B and IL-6. Immunohistochemical examination of NF- κ B expression revealed apparent upregulation in the expression of the inflammatory mediator following DOX intoxication as shown by the intense brown staining (Fig. 10B and G). On the contrary, co-treatment with either NAM or 1 α (OH)D₃ intensely downregulated NF- κ B expression as shown by the faint brown staining (Fig. 10C, D and G). In parallel, the nuclear content of NF- κ B showed a significant rise by 143% in DOX group as compared with the

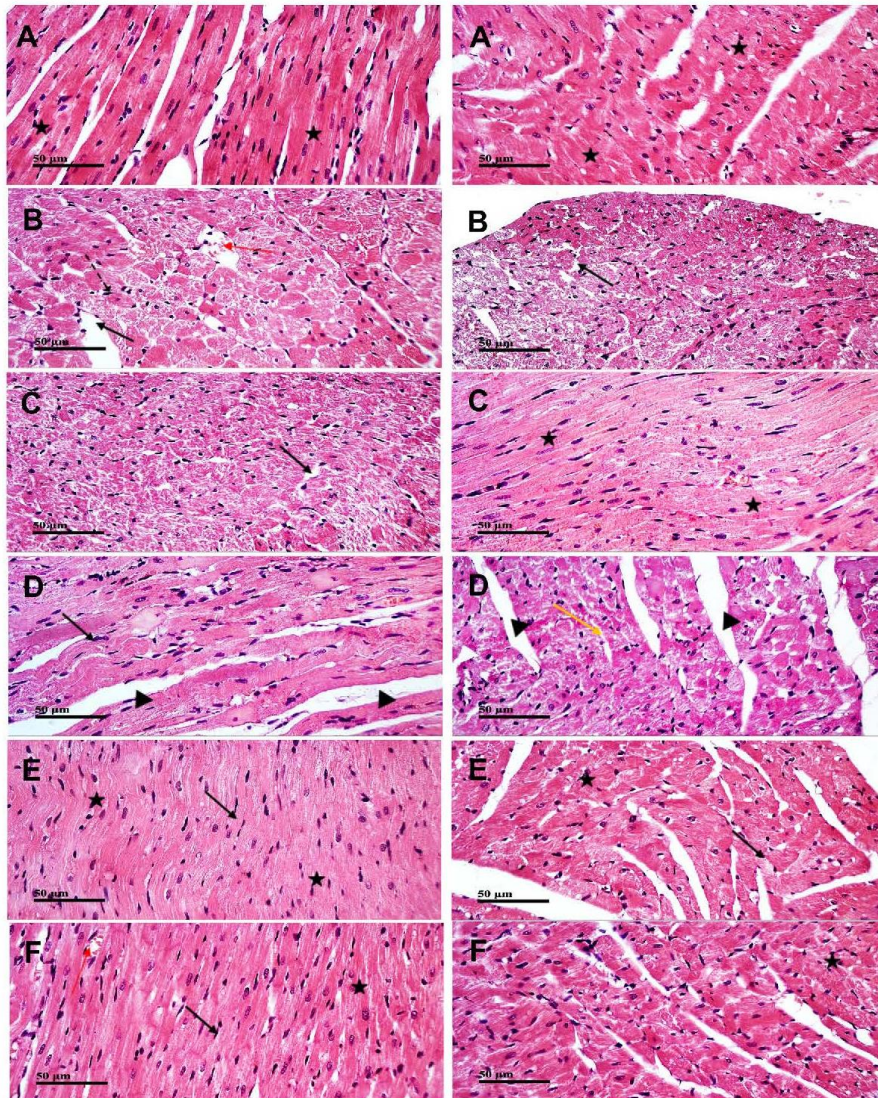
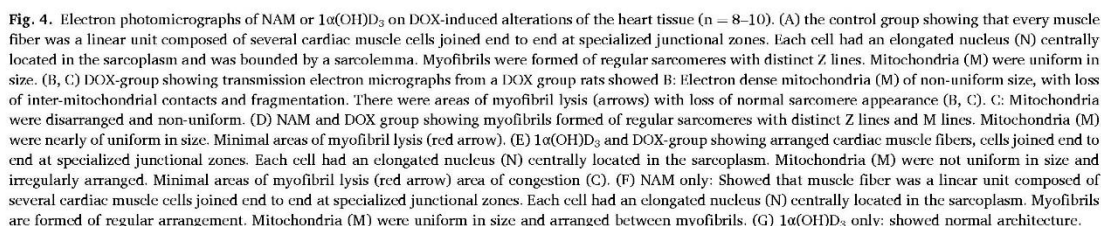


Fig. 3. Effect of NAM or $1\alpha(\text{OH})\text{D}_3$ on DOX-induced histological alterations of the heart tissue (n = 8–10). (A) Control group: demonstrated normal morphological features of different cardiac wall with apparent intact endocardium, well organized branched striated myocardial muscle fibers (star) and delicate epicardium with intact vasculatures, (B) DOX treated group (20 mg/kg) showed focal subendocardial necrosis of cardiomyocytes (arrow) with patches of fragmented cardiomyocytes (arrow) and degenerated fibers with nuclear pyknosis (dashed arrow) accompanied with moderate congestion of intermuscular blood vessels (red arrow), (C) NAM (600 mg/kg) and DOX (20 mg/kg) treated group: showed persistence of alternated apparent intact cardiomyocytes (star) and degenerated and fragmented muscle cells (arrow) with normal intercellular spaces and minimal inflammatory cells infiltrates, (D) $1\alpha(\text{OH})\text{D}_3$ (0.5 $\mu\text{g}/\text{kg}$) and DOX (20 mg/kg) treated group showed persistence of wide areas of degenerative changes of cardiomyocytes (arrow) with higher intercellular spaces, mild hyalinization (arrow head) and few interstitial inflammatory cells infiltrates (yellow arrow), (E) NAM treated group: showed almost intact cardiomyocytes (star) and vasculatures with minimal records of degenerative changes (arrow) and (F) $1\alpha(\text{OH})\text{D}_3$ treated group: showed many intact well organized cardiomyocytes with intact vesicular nuclei (star) and few records of degenerated cardiac cells (arrow) with few congested intermuscular BVs (red arrow).

control group. Interestingly, NAM+DOX-treated group showed a significant reduction in nuclear level of NF- κB by 19% as compared with the DOX group. $1\alpha(\text{OH})\text{D}_3$ + DOX-treated rats showed a non-significant decrease in the nuclear level of NF- κB when compared with the DOX

(Fig. 10H). NAM+DOX-treated rats showed a more significant reduction by 14% over $1\alpha(\text{OH})\text{D}_3$ + DOX-treated animals.

In linear with NF- κB findings, DOX also provoked a significant rise in IL-6 expression in cardiac tissues by 303% as compared with the control



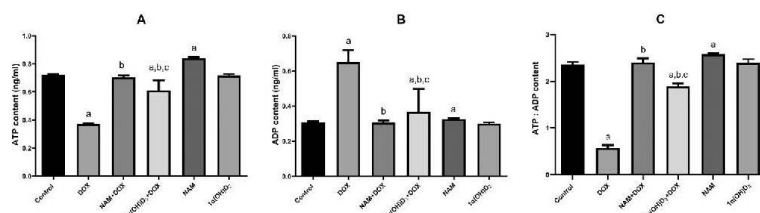


Fig. 5. Effect of NAM or $1\alpha(\text{OH})\text{D}_3$ on cardiac adenosine triphosphate (ATP), adenosine diphosphate (ADP) and ATP:ADP ratio in DOX-induced cardiotoxicity. (A) shows cardiac ATP content, (B) shows cardiac ADP content, (C) shows cardiac ATP:ADP ratio. Values are means \pm SD (n = 8–10). a,b,c: statistically significant from control, DOX or NAM + DOX, respectively at $P < 0.05$ using one-way ANOVA followed by Tukey-Kramer Post Hoc test.

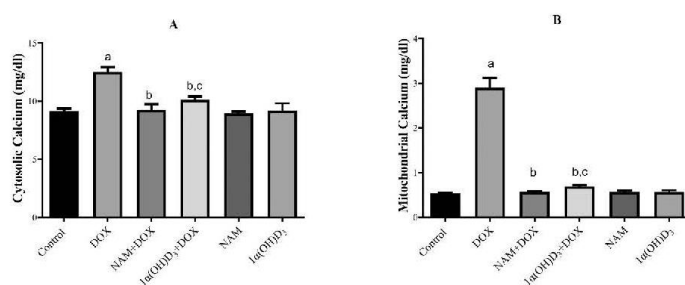


Fig. 6. Effect of NAM or $1\alpha(\text{OH})\text{D}_3$ on Cytosolic calcium (Ca^{2+}_i) and Mitochondrial Calcium (Ca^{2+}_m) in cardiac tissue in DOX-induced cardiotoxicity. (A) shows Cytosolic calcium (Ca^{2+}_i), (B) shows Mitochondrial Calcium (Ca^{2+}_m). Values are means \pm SD (n = 8–10). a,b,c: statistically significant from control, DOX or NAM+DOX, respectively at $P < 0.05$ using one-way ANOVA followed by Tukey-Kramer Post Hoc test.

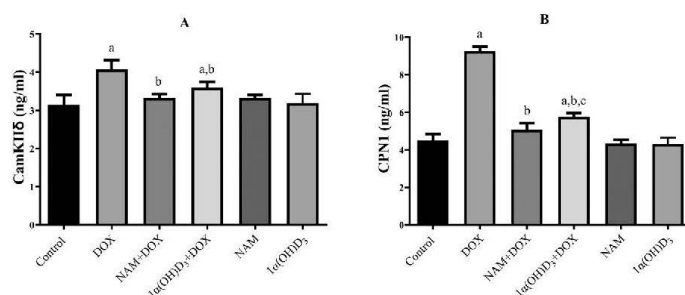


Fig. 7. Effect of NAM or $1\alpha(\text{OH})\text{D}_3$ on calmodulin kinase II gamma (CAMKIIδ) and calpain (CPN1) in DOX-induced cardiotoxicity. (A) shows CAMKIIδ content in cardiac tissue, (B) shows CPN1 content in cardiac tissue. Values are means \pm SD (n = 8–10). a,b,c: statistically significant from control, DOX or NAM+DOX, respectively at $P < 0.05$ using one-way ANOVA followed by Tukey-Kramer Post Hoc test.

group. Conversely, cotreatment of intoxicated animals with either NAM or $1\alpha(\text{OH})\text{D}_3$ + DOX significantly attenuated the IL-6 levels by 68% and 67%, respectively, compared with the DOX treated group (Fig. 10I). It was obvious that the NAM treated group showed more improvement in NF- κ B expression than in the $1\alpha(\text{OH})\text{D}_3$ treated group. Meanwhile, no significant change was detected in cardiac IL-6 between the two groups.

4. Discussion

Cumulative cardiotoxicity is the most devastating complication associated with DOX therapy [24]. Our aim was to investigate and compare the possible cardioprotective effects of NAM and $1\alpha(\text{OH})\text{D}_3$ against DOX-induced cardiotoxicity. In this study DOX cardiotoxicity was characterized by conduction abnormalities and a significant elevation in the activities of the serum cardiac enzymes. These

biochemical data reflected a severe DOX-induced myocardial injury that was verified by a histopathological examination of cardiac tissues and electron microscopy ultrastructure examination of the cardiac myocytes. On the other hand, DOX affected the mortality of animals by 20%. Nicotinamide (NAM) is the amide form of vitamin B₃ [25]. It is considered as the biosynthetic precursor to nicotinamide adenine dinucleotide (NAD⁺), nicotinamide adenine dinucleotide phosphate (NADP⁺) and reduced forms (NAD(P)H) [26]. NAD⁺ derived cofactors are central to cellular homeostasis as they play a crucial roles in intermediary metabolism, mitochondrial respiration, the Krebs' cycle, ATP production and Ca^{2+} signaling [27]. On the other hand, vitamin D is a steroid hormone derived from cholesterol that is recognized as an important substance for maintaining serum Ca^{2+} homeostasis and bone mineralization [28]. Cellular proliferation, differentiation, reduction of inflammation and immune modulation has been linked to the role of

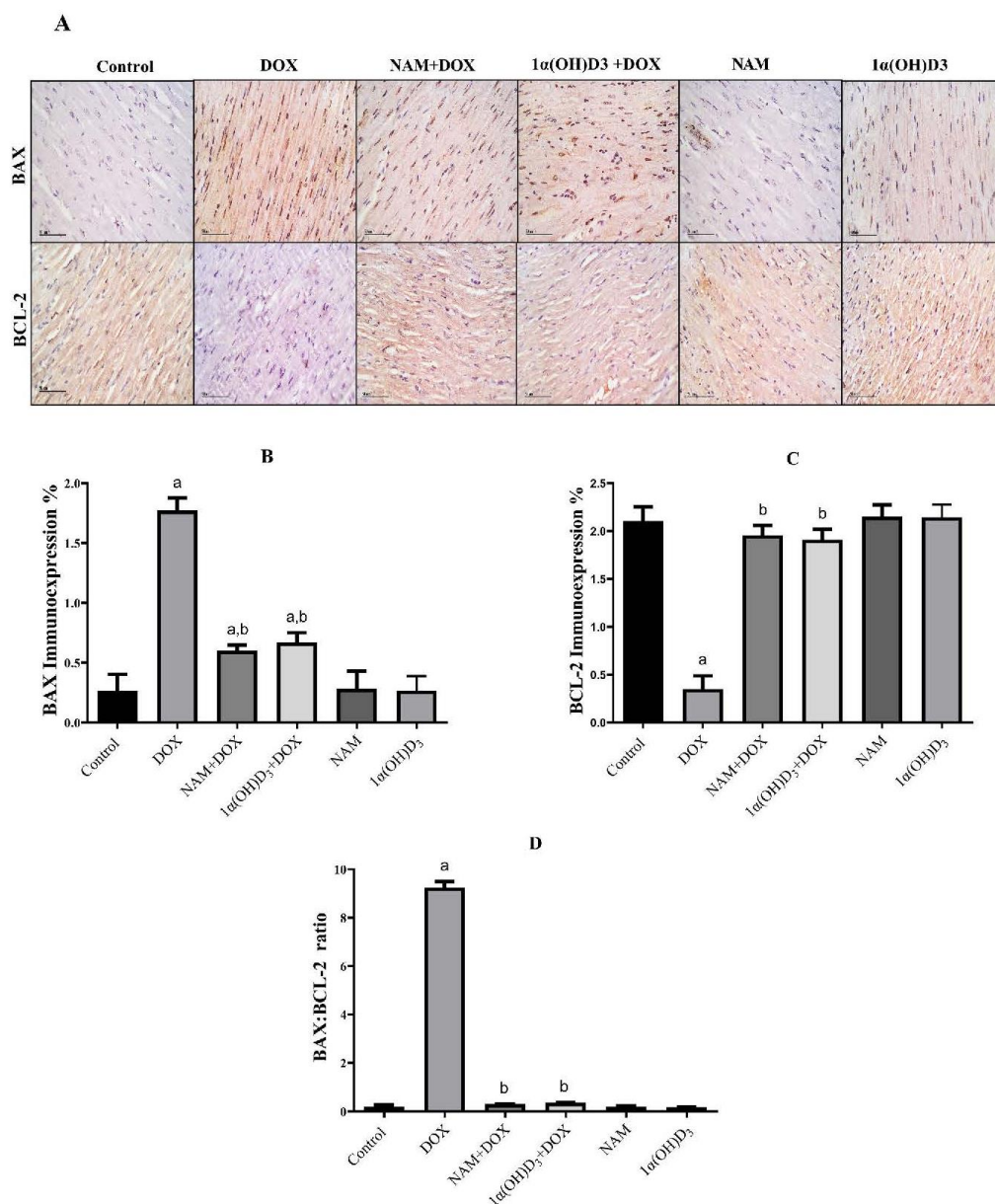


Fig. 8. Effects of NAM or 1α(OH)D₃ on cardiac apoptotic markers (40x) in DOX-induced cardiotoxicity. A: Immunohistochemical staining of cardiac BAX and Bcl-2 expression. B and C: Quantitative image analysis for BAX and Bcl-2 immunohistochemical staining expressed as mean area percent. D: Quantitative image analysis for BAX:Bcl-2 ratio. Data are presented as mean ± SD (n = 8–10). a,b,c: statistically significant from control, DOX, NAM + DOX or 1α(OH)D₃ + DOX, respectively at P < 0.05 using one-way ANOVA followed by Tukey-Kramer Post Hoc test.

vitamin D in human body.

Our study showed that NAM and 1α(OH)D₃ significantly preserved cardiac conductivity and biochemical cardiac markers and preserved the normal architecture of cardiomyocytes indicating the promising cardioprotective effects of both NAM and 1α(OH)D₃ against DOX-induced

myocardial injury. Our results are consistent with previous studies reporting the promising cardioprotective properties of both vitamins in various experimental models of cardiovascular disorders [20,29,30]. Moreover, both vitamins improved the survival rate of the treated animals.