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Study Possible Protective Effects of Naringin and Vitamin D3 On Doxorubicin Induced Cardiac toxicity In Male Rats

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Abstract

This study examined the protective effects of naringin (NRG) and Vitamin D3 (Vit D3) against cardiac toxicity caused by doxorubicin (DOX) in male albino rats. A total of fifty rats were housed individually and divided into five groups: a control group, a DOX group, and groups treated with NRG, Vit D3, or a combination of both. After a two-week treatment period, researchers collected blood samples to analyze various biochemical markers, including cardiac Troponin I (cTnI), Creatine Kinase (CK), and inflammatory and oxidative stress indicators. The DOX group exhibited significantly elevated levels of cTnI, CKMB, and markers such as TNF-α and Caspase-3, reflecting notable cardiac damage. Additionally, levels of Malondialdehyde (MDA) were increased, while antioxidant enzymes like Superoxide Dismutase (SOD) and Catalase were reduced. In contrast, both NRG and Vit D3 treatments significantly decreased the elevated markers of cardiac injury and oxidative stress. They also improved the activity of antioxidant enzymes, suggesting a protective effect against oxidative damage. Histopathological analysis further demonstrated significant improvements in heart tissue structure in the NRG and Vit D3 groups compared to the DOX group. The findings indicate that the co-administration of NRG and Vit D3 provides enhanced protection against DOX-induced cardiotoxicity, suggesting their potential as therapeutic agents to mitigate heart damage in chemotherapy patients.

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Introduction

Adriamycin, another name for doxorubicin (DOX), is a common chemotherapy drug used to treat a variety of cancers. In the 1960s, it was initially separated from the bacteria Streptomyces peucetius var. caesius and has since become a crucial component of cancer treatment protocols (1).

DOX operates by inserting itself between deoxyribonucleic acid (DNA) strands and blocking the activity of topoisomerase II, which is responsible for DNA replication and repair. Nevertheless, this disruption of DNA function can result in double-strand breaks and DNA damage, subsequently triggering pathways that lead to cell death (2).

Although DOX is a highly successful treatment for different types of cancer, its usage is restricted due to the significant negative consequences it can cause. These include irreversible cardiac damage, an increased risk of developing new cancers, hepatotoxicity, nephrotoxicity, suppression of the bone marrow, and harm to the reproductive organs (3).

The rate of doxorubicin-induced cardiotoxicity (DIC) is dose-dependent. It has an incidence of approximately 4% at a dose of 500–550 mg/m², 18% at 551–600 mg/m², and 36% when the dose exceeds 600 mg/m² (4).

Naringin (NRG) is a flavonoid glycoside, belonging to the flavanone class. It is responsible for the bitter flavor of grapefruits; it is abundantly found in grapefruits, especially in the peel and pulp, and found in other citrus fruits, such as oranges and lemons (5).

NRG has demonstrated anti-inflammatory properties that can help mitigate inflammation in the cardiovascular system. Chronic inflammation is linked to the development of cardiovascular diseases, ranging from endothelial dysfunction to clinical events such as myocardial infarction and stroke (6).

NRG has been found to have lipid-lowering effects, including reduction of total levels of cholesterol, LDL, and triglyceride levels. High levels of these lipids are risk factors for cardiovascular diseases (7).

Cholecalciferol, another name for vitamin D3, is a lipid-soluble vitamin that is essential for several physiological functions (8) .

Sunlight exposure is the main source of vitamin D3. When the skin becomes exposed to ultraviolet B (UVB) radiation, a naturally occurring substance called 7-dehydrocholesterol performs a biological process. 7-dehydrocholesterol is changed into provitamin D3 by this reaction, and it subsequently goes through a thermal isomerization step to become vitamin D3(9).

Emerging research suggests that Vit D3 plays a role in modulating immune function. It helps regulate the activity of several immune cells, such as macrophages, T cells, and B cells, essential for recognizing and fighting infections. It also helps maintain immune balance and prevent overactive immune responses (10).

To our knowledge, no prior research has assessed the effect of a combination of NRG and Vit D3 on DIC. So, the present work proposed to evaluate the impact of Doxorubicin on male rats' cardiac tissue and the possible protective effect of NRG and Vit D3.

2. Materials and methods

2.1. Animals:

Fifty male albino rats of the local strain, weighing between 200 and 250 grams, were used in this investigation. They were brought from Tanta University of Medical Sciences' animal house. Five rats per cage were housed in hygienic cages with a 12-hour light-dark cycle and a proper temperature of 23±2ºC. Food and water were freely available to the animals. The Tanta University Faculty of Medicine's ethical committee gave its approval to the experimental protocol.

2.2. Drug and chemicals:

DOX, NRG, and Vit D3 were obtained from (Sigma Aldrich Co). Both DOX and NRG were dissolved in saline but Vit D3 was dissolved in corn oil. Bio-Diagnostic Co. provided all of the kits utilized to measure the parameters in our study.

2.3. Experimental design:

Randomly, the animals were divided into five groups (Ten rats per group) as follows:

1- Group I (Normal control group): the rats in this group received 2.5 ml/kg of normal saline and .01 ml/kg of corn oil daily for two weeks via the intraperitoneal (IP) route.
2- Group II (DOX- received group): the rats received 2.5 mg/kg of Dox three times a week for two weeks via the IP route, resulting in a total cardiotoxic dose of 15 mg/kg (11).
3- Group III (NRG-treated group): rats in this group received 50 mg/kg of NRG per day via the IP route throughout the experiment (12), in addition to DOX at a dosage of 2.5 mg/kg three times a week for two weeks via the IP route (11).
4- Group IV (Vit D3-treated group): This group received DOX at a dosage of 2.5 mg/kg three times a week for two weeks via the IP route (11). Additionally,0.5 µg/kg of Vit D3 was administered every day through the IP route (13).
5- Group V (NRG + Vit D3 treated group): This group received 2.5 mg/kg DOX three times a week for two weeks via the IP method (11),0.5 µg/kg Vit D3 was administered daily via the IP route (13), and 50 mg/kg of NRG was given every day via the IP route (12).

2.4. Blood collection and biochemical analysis:

By the end of the experiment, after a duration of two weeks, the animals were euthanized via cervical decapitation, and blood samples were obtained. The blood was collected and centrifuged at 3000 rpm for 10 minutes. The resulting serum was transferred into clean storage tubes for the assessment of the following parameters: Serum Cardiac Troponin I (cTnI) (14), Serum Creatine Kinase (CK) (15), Serum Tumor Necrosis Factor Alpha (TNF-α) by ELSIA (16), Serum Caspase-3 Analysis by ELISA (17), Serum Nitric Oxide (NO) Analysis (18), Serum Lactate Dehydrogenase (LDH) (19), serum Cytochrome c (Cyt c) (20), serum catalase according (21), serum Superoxide dismutase (SOD) (22).

2.5. Tissue examination:

After dissection, the basal part of the heart was homogenized in a suitable buffer to measure Malondialdehyde (MDA). (23). Histopathological

examination of the heart by light microscope (LM) (Smith,1961), and electron microscope (EM)(24).

2.6 Statistical analysis:

The data were presented as the mean ± standard deviation. A one-way ANOVA, followed by Tukey's test, was used to analyze the study data and evaluate significance. P-values less than 0.05 were deemed statistically significant. All analyses were conducted using SPSS for Windows (Version 26.0).

3. Results

3.1. cTnI, CKMB, and LDH in all studied groups:

These were presented in Table 1, and Fig. 1: in comparison to the normal control group, there was a significant increase in levels of Serum cTnI, CKMB, and LDH in groups Dox, Dox + NRG, and Dox +Vit D3. It was noticed that, compared to the Dox group, there was a significant decrease in these parameters in groups Dox + NRG, Dox +Vit D3, and Dox + NRG + Vit D3. Also shows that there was a significant reduction in these parameters in the Dox + NRG + Vit D3 group in comparison to Dox + NRG and Dox +Vit D3groups.

Table (1): cTnI, CKMB, and LDH in all studied groups.

Parameters	Control group	Dox group	Dox + NRG group	Dox +Vit D3 group	Dox + NRG + Vit D3 group
cTnI (ng/ml)	.029 ±.006	.966 ± .220[a]	.463 ± .151[a,b]	.503 ± .171[a,b]	.043 ± .031[b,c,d]
CKMB (Iu/L)	1.27 ± 0.24	7.02 ± 2.48[a]	4.07 ± 1.22[a,b]	4.35 ± 1.32[a,b]	1.86± 0.39[b,c,d]
LDH (u/l)	188.4 ± 48.5	664.7 ± 172.5[a]	453.0 ± 81.7[a,b]	432.0 ± 96.9[b,c]	247.8 ±89.5[b,c,d]

Data are presented as mean ± SD. Significance levels are indicated as follows: 'a' P< 0.05 compared to the control group, 'b' P < 0.05 compared to the (Dox) group, 'c' P < 0.05 compared to the (Dox + NRG) group, and 'd'P < 0.05 compared to the (Dox + Vit D3) group.

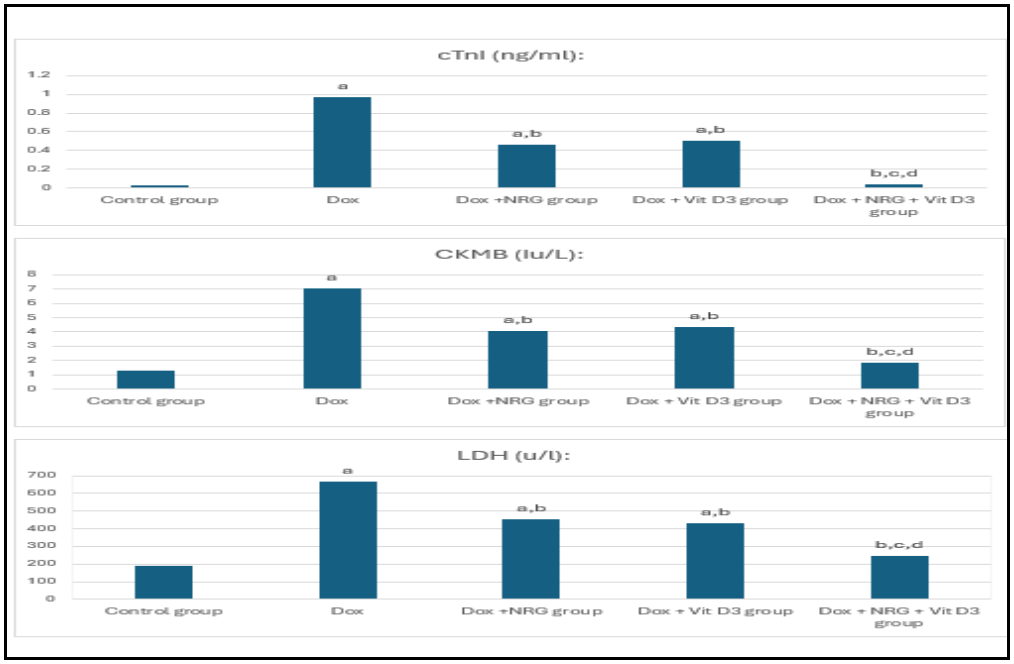

Figure (1): cTnI, CKMB, and LDH in all groups: Data are presented as mean ± SD. Significance levels are indicated as follows: 'a' P< 0.05 compared to the control group, 'b' P < 0.05 compared to the (Dox) group, 'c' P < 0.05 compared to the (Dox + NRG) group, and 'd' P < 0.05 compared to the (Dox + Vit D3) group.

3.2. SOD and Catalase in all groups:

As shown in Table 2 and Figure 2, the Dox group's levels of SOD and Catalase were significantly lower than those of the normal control group. All of these metrics were found to be significantly higher in other groups than in the Dox group.

Table (2): SOD and Catalase in the studied groups.

Parameters	Control group	Dox group	Dox + NRG group	Dox +Vit D3 group	Dox + NRG + Vit D3 group
SOD (Iu/L)	32.12 ± 3.63	14.21 ± 4.19^{a,b}	21.09 ± 5.99^{a,b}	20.92 ±2.57^{a,b}	28.31±4.93^{b,c}
Catalase (nmol/ml)	21.1 ± 2.6	7.9 ± 2.5 ^{a}	17 ± 2 ^{b,d}	17 ± 2^{a,b,c}	21.5± 1.2^{b,d}

Data are presented as mean ± SD. Significance levels are indicated as follows: 'a' P< 0.05 compared to the control group, 'b' P < 0.05 compared to the (Dox) group, 'c' P < 0.05 compared to the (Dox + NRG) group, and 'd'P < 0.05 compared to the (Dox + Vit D3) group.

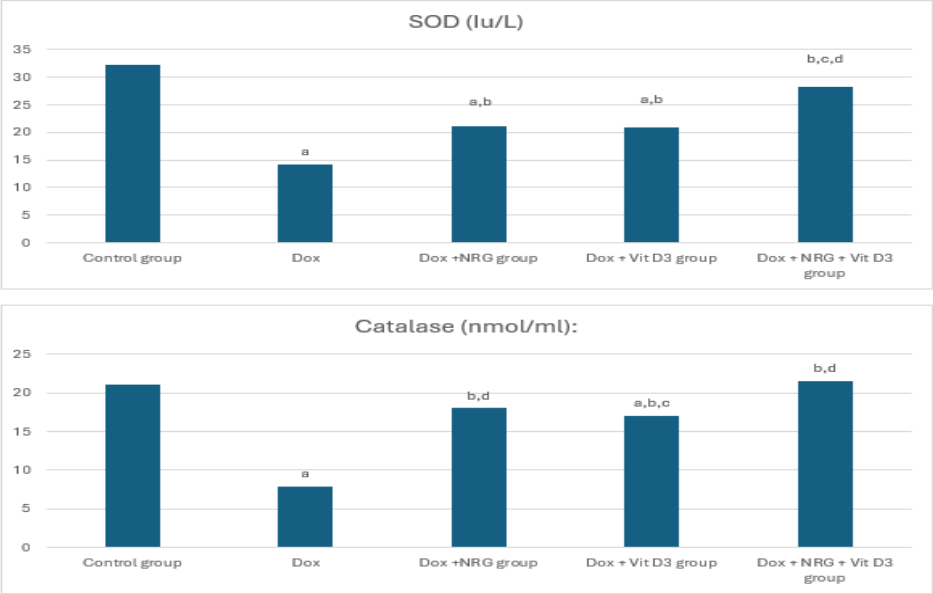

Figure (2): SOD and Catalase in all groups. Data are presented as mean ± SD. Significance levels are indicated as follows: 'a' P< 0.05 compared to the control group, 'b' P < 0.05 compared to the (Dox) group, 'c' P < 0.05 compared to the (Dox + NRG) group, and 'd' P < 0.05 compared to the (Dox + Vit D3) group.

3.3. Serum NO and Cardiac MDA in all groups.

Serum NO and Cardiac MDA levels were increased significantly in the group that received Dox in comparison to the control group. While in the NRG or Vit D3 treated groups, the NO and MDA levels were significantly decreased if compared to the Dox group. The levels of NO and MDA were nearly like the normal levels in the NRG +Vit D3 group, table 3 & fig.3.

Table (3): Serum NO and Cardiac MDA in all studied groups.

Parameters	Control group	Dox group	Dox + NRG group	Dox +Vit D3 group	Dox + NRG + Vit D3 group
NO (µmol/L)	16.10 ± 5.14	44.52 ± 8.71[a]	30.69 ± 5.85 [a,b]	31.74±10.43[a,b,c]	18.26 ± 6.55[b,c,d]
MDA (nmol/mg)	.99 ± .28	3.06 ± .60 [a]	1.46 ± .45 [b]	2.09 ± .85[a,b]	1.16 ± .33 [b,d]

Data are presented as mean ± SD. Significance levels are indicated as follows: 'a' P< 0.05 compared to the control group, 'b' P < 0.05 compared to the (Dox) group, 'c' P < 0.05 compared to the (Dox + NRG) group, and 'd'P < 0.05 compared to the (Dox + Vit D3) group.

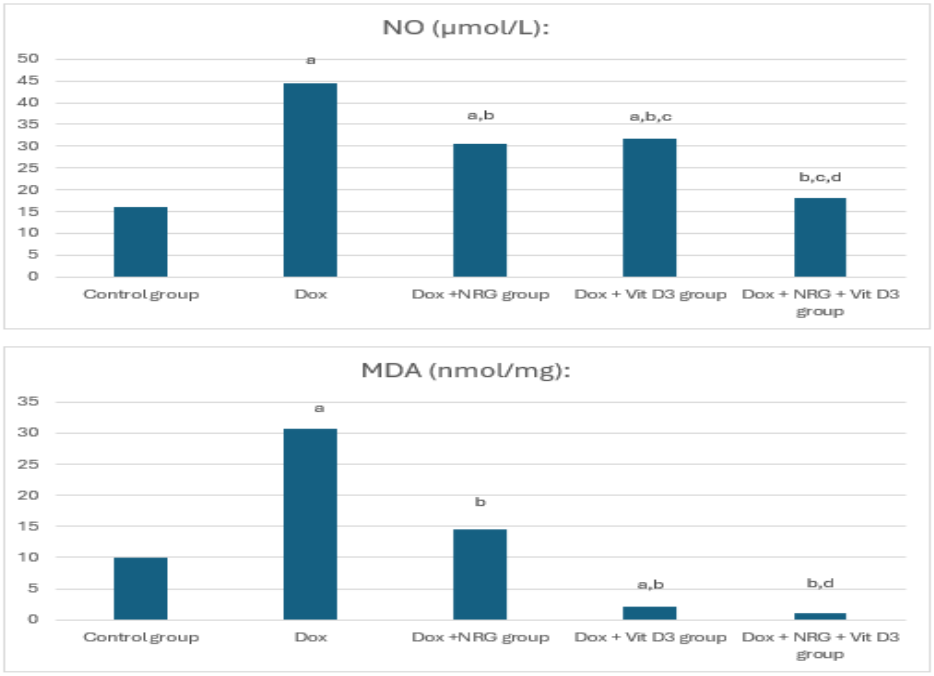

Figure (3): Serum NO and Cardiac MDA in all studied groups.

Data are presented as mean ± SD. Significance levels are indicated as follows: 'a' P< 0.05 compared to the control group, 'b' P < 0.05 compared to the (Dox) group, 'c' P < 0.05 compared to the (Dox + NRG) group, and 'd'P < 0.05 compared to the (Dox + Vit D3) group.

3.4. Serum TNF, Caspase 3, and Cytochrome C in all studied groups.

Serum TNF, Caspase 3, and Cytochrome C levels showed a significant rise in the Dox group compared to the control group. While in NRG or Vit D3 treated groups, the Serum TNF, and Caspase 3 levels showed a significant decrease compared to the Dox group. The levels of Serum TNF and Caspase 3 were returned to normal levels in the NRG + Vit D3 group Table4& fig.4.

As regards Cytochrome C level, it was significantly decreased in the NRG group as compared to the Dox group. However, there were no significant changes in its level in the Vit D3 group in comparison to the Dox received group, Table 4& Fig. 4.

Table (4): Serum TNF, Caspase 3,and Cytochrome C in the studied groups.

Parameters	Control group	Dox group	Dox + NRG group	Dox +Vit D3 group	Dox + NRG + Vit D3 group
TNF (Pg/ml)	101.1±10.1	429.9 ±89.8 [a]	267.0± 23.4 [b]	301.6± 17.6[a,b]	146.8 ± 27.2[b,d]
Caspase 3 (ng/ml)	.164± .050	1.632± .599 [a]	.951 ± .138 [a,b]	.793 ± .328 [a,b]	.264 ± .068 [b,c,d]
CytochromeC (ng/ml)	24.13±7.82	43.38 ± 4.31 [a]	23.89 ± 4.21 [b]	40.11 ± 4.25 [a,c]	25.31 ± 4.74 [b,d]

Data is presented as mean ± SD. The significance levels are as follows: 'a' $P < 0.05$ compared to the control group, 'b' $P < 0.05$ compared to the (Dox) group, 'c' $P < 0.05$ compared to the (Dox + NRG) group, and 'd' $P < 0.05$ compared to the (Dox + Vit D3) group.

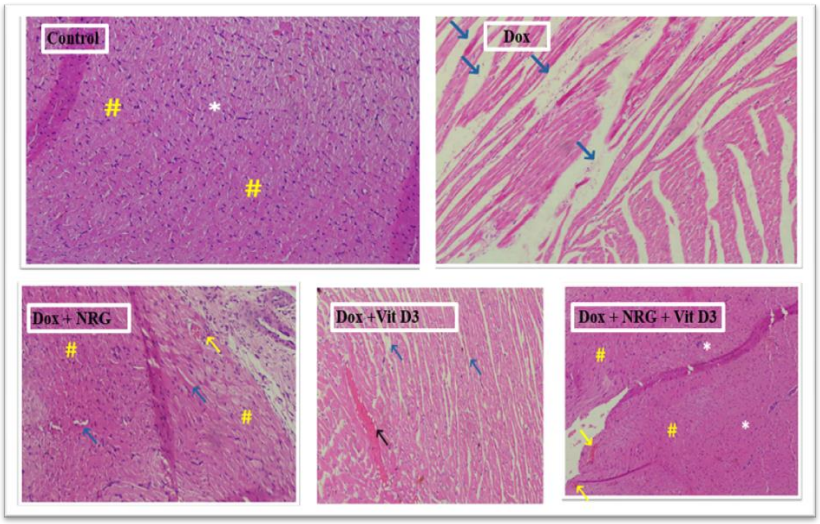

Figure (5): Histopathological analysis of all groups using a light microscope (LM).

The control group showed a typical cardiac structure with pink-stained myocytes and darkly stained nuclei (represented by a white star *). Striated muscle fibers are also visible (represented by a yellow #). No signs of edema, necrosis, or hemorrhage are seen. The Dox group, in comparison to the control group, showed loss of striations and extensive myolysis (represented by a blue arrow) Compared to the Dox group the NRG group showed preserved striations (represented by a yellow #), mild myolysis (represented by a blue arrow), and a few areas of (represented by a yellow arrow).Also, the Vit D3 group showed a moderate degree of myolysis (represented by a blue arrow), and few cytoplasmic vacuolations (represented by a black arrow), with no signs of hemorrhage. The group treated by NRG + Vit D3 showed mostly normal myocytes with prominent nuclei (represented by a white star *), preserved striation (represented by a yellow #), and scattered areas of hemorrhage (represented by a yellow arrow).

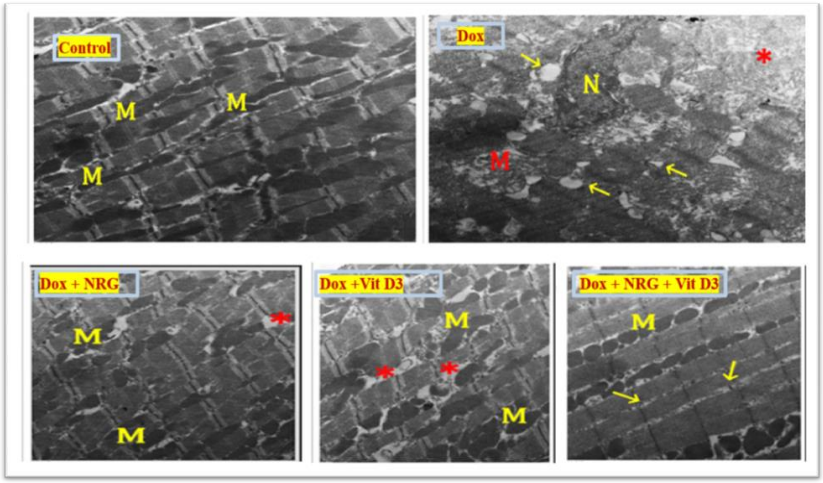

Figure (6): Histopathological analysis of all groups using electron microscopy (EM).

The control group showed a normal cardiac structure with a normally appeared nucleus and preserved sarcomere with regularly spaced Z-lines and normally arranged T tubules, also showed abundant mitochondria, arranged in rows between myofibrils (represented by M).

Compared to the normal group, the Dox group showed abnormal structure of cardiac tissue as severe myolysis (represented by red star *), loss of arranged cardiac sarcomere, an abnormal nucleus with no nucleolus (represented by N), and abnormal mitochondria (represented by M) with cytoplasmic vacuolations (represented by yellow arrow).

Compared to the Dox group, the NRG group showed preserved sarcomere, multiple mitochondria (represented by M), and a mild degree of myolysis (represented by a red star). Also, the Vit D3 group showed multiple normal-sized mitochondria (represented by M), a preserved sarcomere with arranged Z lines, and a moderate degree of myolysis (represented by red star *),

The NRG and Vit D3 combination appeared in a nearly normal cardiac structure with normally arranged mitochondria (represented by M), preserved sarcomere with prominent Sacro tubules (represented by yellow arrow), and normal cytoplasm without vacuolations.

4. Discussion

In the current work, Dox-induced cardiotoxicity was manifested by increased serum levels of cardiac biomarkers like cTnI, CKMB, and LDH. Meanwhile, the NRG and Vit D3 attenuated the inflammatory conditions of DOX as proved by reduced serum levels of cTnI, CKMB, and LDH. Suggesting their therapeutic potential against cardiotoxicity.

Also, it was observed from our results that a combination of NRG with Vit D3 was more effective than each drug alone as shown by significant improvement of all studied parameters.

Our experiment showed that in the DOX group, there was a significant increase in the cTnI, CKMB, and LDH compared to the control group. Treatment with either NRG or Vit D3 significantly reduced the cTnI, CKMB, and LDH which is explained by anti-inflammatory actions and enhancement of antioxidant defenses and immunosuppressive effects of them. The combination of NRG and Vit D3 demonstrated a synergistic effect in lowering cardiac biomarker levels more effectively than either treatment alone.

We also proved that the serum level of NO and cardiac MDA was significantly higher in the Dox group than in the normal control group. Also, there was a significant decrease in antioxidant enzymes like SOD and catalase which illustrate the oxidative stress caused by DOX.

Also, our results showed that there was a significant decrease in serum level of NO and cardiac MDA and a significant increase of antioxidant enzymes like SOD and catalase in the group treated by NRG or Vit D3 which demonstrates the possible antioxidant effect of NRG and Vit D3.

Like our results, Gelen and Şengül, (2020) (25) found that NRG might protect against cisplatin-induced heart damage by decreasing cardiac oxidative stress by increasing SOD.

One of the possible mechanisms is that NRG was shown to upregulate the gene expression and

activity of SOD, helping to mitigate oxidative stress in cases of ischemia-reperfusion injury (26).

Like our findings, recent studies discovered that Vit D3 supplementation could increase SOD activity (27). Thismay be because of the role of Vit D3 in the activation of the Nuclear Factor Erythroid 2-related Factor 2 (NRF2), which is responsible for regulating the expression of antioxidant genes, including SOD and catalase (28).

According to our findings, the DOX-treated group showed significantly increased levels of TNF and caspase 3 in comparison with the control group, which in turn demonstrated cardiac cell injury inflammation and apoptosis by Dox.

Our observations also revealed a significant decrease in the level of TNF and caspase 3 in the groups treated by NRG or Vit D3 compared to the DOX-treated group, suggesting that NRG or Vit D3 may play a health-promoting function in avoiding cardiotoxicity.

One of the probable mechanisms is the ability of NRG to inhibit gene expression of TNF and caspase 3 (29).

In the same line, the study by Wang et al., (2019) (30) demonstrated the chemoprotective effect of NRG in breast cancer through suppression of caspase-3 and TNF.

Additionally, a study by Fathi et al., (2022) (31) on rats with streptozotocin-induced diabetes illustrated that rats that received Vit D3exhibited a significant reduction in caspase-3 activity compared to non-treated rats.

Our study revealed that the combination of both NRG and Vit D3 was better than each drug alone illustrated by the significant decrease in levels of TNF and caspase 3 if compared with other treated groups.

Based on our research findings, compared to the control group, the DOX-treated group had a significantly higher level of Cyt c. The current investigation also showed a considerable drop in serum Cyt c levels in the NRG group compared to the DOX group. Recent research suggested that NRG exerts its anti-apoptotic effects by inhibiting the release of Cyt c from mitochondria to the cytoplasm, thereby preventing the activation of the mitochondrial apoptotic pathway (32). Consistent with our research Garaba du and Agrawal, (2020) (33) discovered that NRG could decrease serum Cyt c, preventing intrinsic apoptosis in Rotenone induced neurotoxicity in Experimental Rodents. Our findings revealed no significant changes between the DOX and Vit d3 treated groups, implying that Vit d3 may promote cytochrome c expression, resulting in apoptosis. Like our findings, Na et al., (2022) (34) discovered that Vit d3 can activate caspase 3, leading to apoptosis by boosting Cyt C expression.

The light microscope (LM) analysis revealed that DOX-treated myocytes showed significant myolysis and disrupted striations compared to the control group, indicating DOX's direct cytotoxicity. These changes, including increased vacuolization, suggest alterations in mitochondrial structure and function, aligning with findings on DOX-induced oxidative stress.

In contrast, NRG-treated myocytes exhibited better-preserved morphology, with reduced vacuolization and organized myofibrils, suggesting NRG's protective effect on mitochondrial integrity due to its antioxidant properties as proved by Zhao et al., (2022) (35) who illustrated the antioxidant

and mitochondrial-stabilizing properties of NRG, which may have counteracted the DOX-induced oxidative stress and mitochondrial dysfunction. Similarly, LM analysis of the Vit D3-treated group showed improved cardiomyocyte architecture, with less vacuolization and better-maintained nuclear morphology, indicating that Vit D3 can mitigate the cardiotoxic impact of Dox.

Our findings align with Saleh et al. (13), which highlighted the protective effects of Vit D3 against DOX's cytotoxic impact on cardiac muscle cells. Additionally, our research indicates that a combination of NRG and Vit D3 offers greater protection against DOX than either drug alone. This is evidenced by observations of mostly normal myocytes with preserved cardiac striations.

The EM analysis of the DOX group revealed significant ultrastructural changes, including severe myolysis, disorganized cardiac sarcomeres, and loss of Z-line integrity, indicating the mechanisms behind DOX cardiotoxicity. Notably, chromatin condensation and nuclear margination were observed, marking apoptosis, with aberrant mitochondria that indicate oxidative stress and compromised mitochondrial activity.

Conversely, the EM analysis of the NRG group showed improved mitochondrial ultrastructure and better-preserved sarcomere organization. This aligns with findings by Yaseen et al. (2024) (35) on NRG's anti-inflammatory and antioxidant effects.

Similarly, the Vit D3 group exhibited enhanced sarcomere organization and fewer autophagic vacuoles, indicating that Vit D3 may have regulated autophagy and mitigated DOX-induced damage. Consistent with Lee et al. (2021) (36), who noted that Vit D3 reduces DOX-induced cardiotoxicity without affecting its anticancer efficacy.

Overall, the combined antioxidant and anti-inflammatory effects of NRG, along with Vit D3's ability to prevent apoptosis, suggest a potential synergistic cardioprotective mechanism, indicating that their combination may effectively reduce the cardiotoxic effects of DOX chemotherapy.

5. **Conclusion:** We demonstrated in this study that the co-administration of NRG and Vit D3 was better than either treatment alone. The powerful antioxidant and anti-inflammatory qualities of NRG combined with Vit D3's capacity to prevent apoptosis appear to be the synergistic cardioprotective mechanism. These findings imply that NRG and Vit D3 combination therapy may be a viable therapeutic approach to reduce the cardiotoxic impact of DOX chemotherapy. So, further investigations will be done to confirm the effectiveness, mechanisms, and possible clinical uses of this combination approach.

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