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Original Article

Protective effects of omega-3, atorvastatin, vitamin E and vitamin C against doxorubicin-induced cardiotoxicity in rats: a comparison study

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Abstract

Introduction: The stress-oxidative is involved in doxorubicin (DOX)-induced cardiotoxicity. Due to the potential and previous reported for antioxidant properties of atorvastatin, omega-3, vitamin E and vitamin C, their efficacy to prevention of DOX-induced cardiotoxicity was investigated in this study

Methods: Fifty-six male rats were divided into 8 groups which received omega-3, atorvastatin, vitamin E, vitamin C, normal saline and dimethyl sulfoxide (DMSO) via gavage for 14 days then a single dose of DOX (20 mg/kg) was injected intraperitoneally except two last groups that received only normal saline or DMSO. The level of oxidative stress parameters like ferric reducing ability of plasma (FRAP) before and after DOX injection and malondialdehyde (MDA) of heart were estimated. Also the histopathologic assessments were done on heart sample at the end of experimental period.

Results: The results showed that compared to other agents, omega-3 could emerge as the most protection against DOX. Its pretreatment led to one of the most FRAP changing percent meanwhile less MDA value and cardio pathologic indexes almost close to control groups compared to that of other agents (P<0.01).

Conclusion: Omega-3 may have a promising protective effect against DOX-induced cardio toxicity.

Keywords:
Doxorubicin;
Omega-3;
Vitamin E;
Vitamin C;
Atorvastatin;
Cardiotoxicity

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Introduction

Doxorubicin (DOX) as a topoisomerase II inhibitor produces double-strand DNA breaks thereby useful for treatment of different tumors (Abdel-Raheem and Abdel-Ghany, 2009); however, it induces the cardiotoxicity and heart failure result in the restricted

clinical use (Nagi and Mansour, 2000). The multi-factorial cardiotoxicity with distinct mechanism of antitumor activity is observed in 2–20% of the patients receiving DOX and accounts for as high as 50% mortality within 2 years treatment (Todorova et al., 2010; Wattanapitayakul et al., 2005). The quinine moiety of DOX molecule along with its metabolite doxorubicinol induce the cardiotoxicity with features like the disruption of myocyte, vacuolization, dilatation of the sarcoplasmic reticulum, mitochondrial injury, electrocardiographic abnormalities (Wold et al., 2005; Yagmurca et al., 2003) and low expression of mitochondrial manganese superoxide dismutase or cysteine-rich metallothioneins (Yoshida et al., 2009). Some of the molecular events engaged in cardiotoxicity contains the generation of reactive oxygen species, lipid peroxidation (Abdel-Raheem and Abdel-Ghany, 2009; Cappetta et al., 2017; Kalender et al., 2002; Shah et al., 2009; Wold et al., 2005; Yagmurca et al., 2003) vasoactive amine release, intracellular Ca^{2+} overload, impairment in myocardial adrenergic signaling/regulation (Wold et al., 2005), induction of iNOS, apoptosis, altered vascular structures (Akolkar et al., 2017; Huelsenbeck et al., 2011), increased the levels of cardiac proinflammatory cytokines (Akolkar et al., 2017) and the disruption of catalytic cycle through topoisomerase 2β (Hsu et al., 2014).

There are some reports inclusive of decreasing chemotherapeutics agents-induced oxidative stress by different antioxidants or free radical scavengers to improve the compliance of patients. That way, four of the most investigated agents have been omega-3, statins, vitamin C and vitamin E. So that, the polyunsaturated fatty acids of omega-3 could reduce the adverse effects of some chemotherapy drugs (Brown et al., 2003; Hardman et al., 2002; Uygur et al., 2014) while enhance their anti-tumor activity (Pardini, 2006). Also, statins pretreatment could attenuate DOX-induced side effects (El-Moselhy and El-Sheikh, 2014; Kalam and Marwick, 2013; Riad et al., 2009). As the same way, ascorbic acid and its derivatives as well known antioxidant and scavenger alleviate the toxicity of DOX delay (Amara-Mokrane et al., 1996; Heaney et al., 2008; Santos et al., 2007; Shimpo et al., 1991). Accordingly, vitamin E has shown the protection against some aspects of DOX-induced damage (Antunes and Takahashi, 1998; Hadi et al., 2012; Puri et al., 2005; Santos et al., 2007; Tsai et al., 2010).

Therefore, similar to other situations, when there are different choices, the selection of agent with more efficiency may be useful. Then despite the multiple evidences regarding to the potential protective effect of interested antioxidants, at the present work their efficacy as the prophylactic administration on DOX-induced cardiotoxicity was compared. Also the intended effects of asked agents have been studied in different and distinct conditions, so the investigation and comparison of their impacts seems to be reasonable. It would be helpful to choice more efficient agent to reduce chemotherapeutics side effects.

Materials and methods

Ethics and animals

Animal studies were conducted according to the guidelines for the care and handling of animals prepared by the Iranian Ministry of Health (ethics board approval number: 90-114-15 Arak University of Medical Sciences) and the internationally accepted principles for laboratory animal use and care as found in the European Community Guidelines (EEC Directive of 1986; 86/609/EEC).

Experimental design

The locally bred male Wistar rats (250-300g) kept under a 12 h light-dark cycle at room temperature and had free access to food and water. Experimental animals were divided into 8 groups (7 rats in each group) as follows: omega-3 (Vitex Pharm Co, Australia) 1 g/kg (Brown et al., 2003); atorvastatin (Sobhan Darou Co, Iran) 100 mg/kg (Riad et al., 2009) dissolved in dimethyl sulfoxide (DMSO, Merck, Germany); vitamin E (Osve Co, Iran) 400 IU/kg (Kalender et al., 2002; Lippman et al., 2009; Puri et al., 2005) and vitamin C (Darou Pakhsh Co, Iran) 250 mg/kg (Wold et al., 2005).

All of the agents were administrated via gavage for 14 days and delivered to the appropriate volume by normal saline (NS) for better administration. Then a single dose (20 mg/kg, intraperitoneally) (Riad et al., 2009; Yagmurca et al., 2003) of DOX (EBEWE, Austria) was taken on 14th day. Due to the usage of NS or DMSO (used at least necessary) along with agents, two further groups were added in which received only NS or DMSO, 2 ml/day for 14 days.

Collection of blood and heart samples

To measurement of ferric reducing ability of plasma (FRAP) value of pre-DOX, the blood samples were taken from the tails on the fourteenth day just before DOX injection. Then 48 h later, the rats were anesthetized by ketamine (Rotexmedica, Germany) and xylazine (Alfasan, Netherlands) and perfused. The hearts were used for measurement of oxidative stress biomarkers and histopathological index of heart (Abdel-Raheem and Abdel-Ghany, 2009; Todorova et al., 2010).

FRAP assay

During FRAP measurement, the concentration of Fe^{2+}-TPTZ (2, 4, 6-Tri (2-pyridyl)-1, 3, 5-triazine) (all of agents, Merck, Germany) was considered as a function of antioxidant ability. The absorbance of samples was read against blank solution at 532 nm by spectrophotometer (Biowave II, Biochrom, Cambridge, England) (Jafarey et al., 2014; Shams Ardekani et al., 2011).

Measurement of malondialdehyde (MDA)

To do the test, these solutions were prepared: the phosphate buffer (NaCl 8g, KCl 0.2g, Na_2HPO_4 1.044g and KH_2PO_4 0.24g), sodium dodecyl sulfate (8.1%), tetraethoxypropane (5, 10, 20 and 40mM), thiobarbituric acid (0.8% solved in Na_2SO_4 2M at 60°C) and 20% acetic acid (its pH 3.5 by NaOH). Thiobarbituric acid produces a pink complex with MDA as a final marker of oxidative stress at high temperature and acidic pH. The measurement of this reaction and its light absorbance is regarded as lipid peroxidation.

After perfusion, the hearts were weighed, kept at -20 °C and then were homogenized (Heidolph, Germany). The tubes were centrifuged (Zentrifugen, Tuttlingen, Germany) and the MDA test was done in accordance to previous studies (Abdel Moneim and El-Khadragy, 2013; Jafarey et al., 2014; Noeman et al., 2011). The light absorbance of upper layer was read at 532 nm by spectrophotometer (Biowave II, Biochrom, Cambridge, England).

Histopathological evaluation

Four micrometer-thick paraffin sections were prepared from fixed heart in 10% paraformaldehyde. After staining with hematoxylin and eosin (Merck, Germany), a minimum of 8 fields for each heart section were assigned to severity of changes by a blinded observer. The evaluated histopathologic indexes of heart were considered like edema, hyalinization, sever congestion, focal hemorrhage, focal necrosis, myofibrillar loss, vacuolization, diffuse cell damage, fibrosis and loss of organelles. All of them were assessed separately as scores so trace, 1+, 2+ and 3+ dependent on the severity of lesion. The total score of pathologic indexes for each group was considered as well (Abdel-Raheem and Abdel-Ghany, 2009; Todorova et al., 2010)

Statistical analysis

The analysis of data was accomplished by SPSS 16. All values of FRAP, MDA and pathologic scores were expressed as mean±SEM. The FRAP and MDA data were analyzed by one-way ANOVA followed by Tukey's test. The FRAP data for each group before and after DOX injection were analyzed by paired t-test. The pathologic scores were analyzed by Kruskal-Wallis test followed by Dunn's multiple comparison test. Spearman correlation was used to evaluate correlation between the various parameters. The significance level was set at $P<0.05$.

Results

Figure 1 shows the plasma level of FRAP (µM) for groups at pre and post-injection of DOX. The results showed that there were significant differences among groups for both of before and after DOX treatment (pre-DOX injection: F (5, 69) =46.692; $P<0.0001$; post-DOX injection: F (5, 65) = 54.2; $P<0.0001$). It also revealed that vitamin C and E induced the most FRAP value compared to other groups significantly ($P<0.001$). It also reveals that omega-3 can significantly increase the plasma FRAP value compared to groups receiving NS, DMSO and atorvastatin after DOX administration ($P<0.001$).

To better comparison of the FRAP changes before and after DOX treatment, the mean±SEM of values and percent of changing for each group are shown in Table 1. It indicates a significant increase of the FRAP value for all of agents except NS after DOX injection. However, omega-3 and atorvastatin could induce the most increase of percent of the FRAP value related to before DOX injection such 236.856% for omega-3 and 340.496% for atorvastatin ($P<0.0001$).

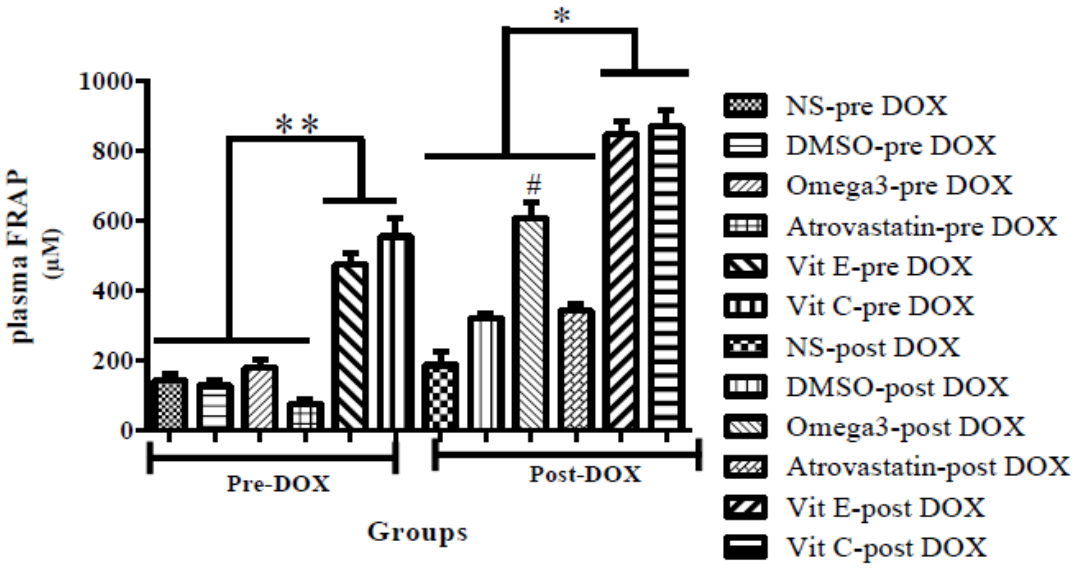

Fig.1. The mean of plasma FRAP micromolar (µM) in the studied groups in pre and post doxorobicin (DOX) injection. **the most significant value related to other groups at before DOX injection (*P*<0.001); *the most significant value than related to other groups at after DOX injection (*P*<0.001); #significant difference related to NS, DMSO and atrovastatin at after DOX injection (*P*<0.001).

NS: Normal saline; DMSO: dimethyl sulfoxide; Pre-DOX: a period when during all of the agents were administrated via gavage for 14 days just before DOX injection; DOX injection: as single dose 20 mg/kg; Post-DOX: 48 h after DOX injection without any agent interference considered for the measurement of oxidative stress biomarkers and histopathological index of heart.

Table 1: The comparison of mean±SEM of plasma FRAP micromolar (µM) in the studied groups before versus after DOX injection.

Plasma FRAP micromolar (µM)				
Group	Pre-DOX injection	Post-DOX injection	Percent of changing	statistics
Normal saline	144.4±17.01	187.7±39.57	29.98615	t(11)=0.9176 P value=0.3785
DMSO	129.3±14.77	322.2±14.44	149.1879**	t(9)=7.138 P<0.0001
Omega3	180.3±22.17	608.7±43.85	236.8567***	t(13)=6.822 P<0.0001
atorvastatin	78.23±11.44	344.6±17.63	340.496***	t(7)=16.133 P<0.0001
Vit E	476.1±33.48	848.8±37.77	78.28187**	t(13)=6.427 P<0.0001
Vit C	556.2±51.93	871.4±45.27	56.67026*	t (11)=5.491 P value=0.004

***most significant percent of FRAP value changing; **significant percent of FRAP value changing; *least significant percent of FRAP value changing

Pre-DOX: a period when during all of the agents were administrated via gavage for 14 days just before DOX injection; DOX injection: as single dose 20 mg/kg; Post-DOX: 48 h after DOX injection without any agent interference considered for the measurement of oxidative stress biomarkers & histopathological index of heart

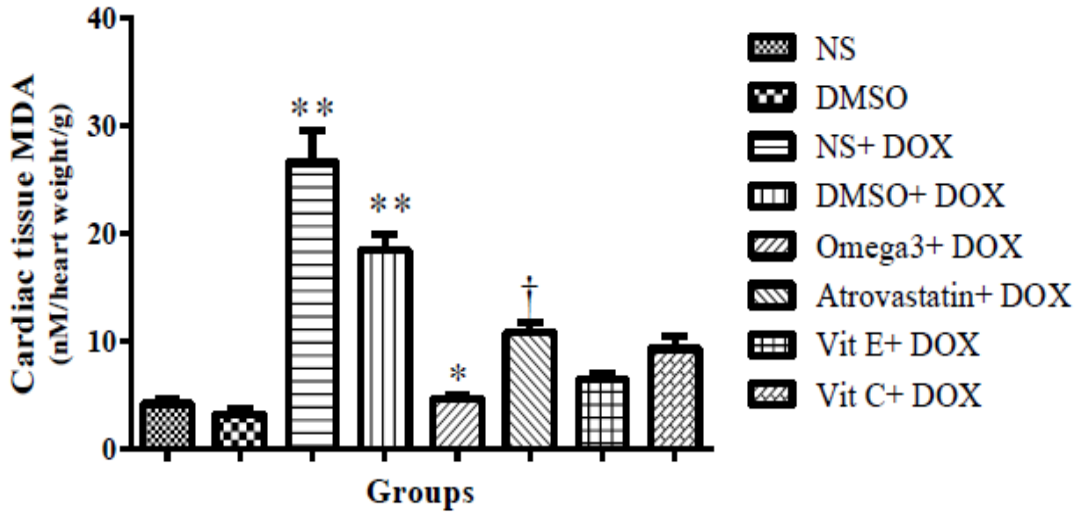

Fig.2. The mean of cardiac tissue MDA (nM/g cardiac weight) in the studied groups. *less significant to atorvastatin+DOX (*P*<0.05); †more significant compared to NS and DMSO (*P*<0.001); **most significant value than that of other groups (*P*<0.001).
nM/g cardiac weight: nanomolar/ g cardiac weight; Agent+DOX: the groups that were taken agents before DOX injection for 14 days.

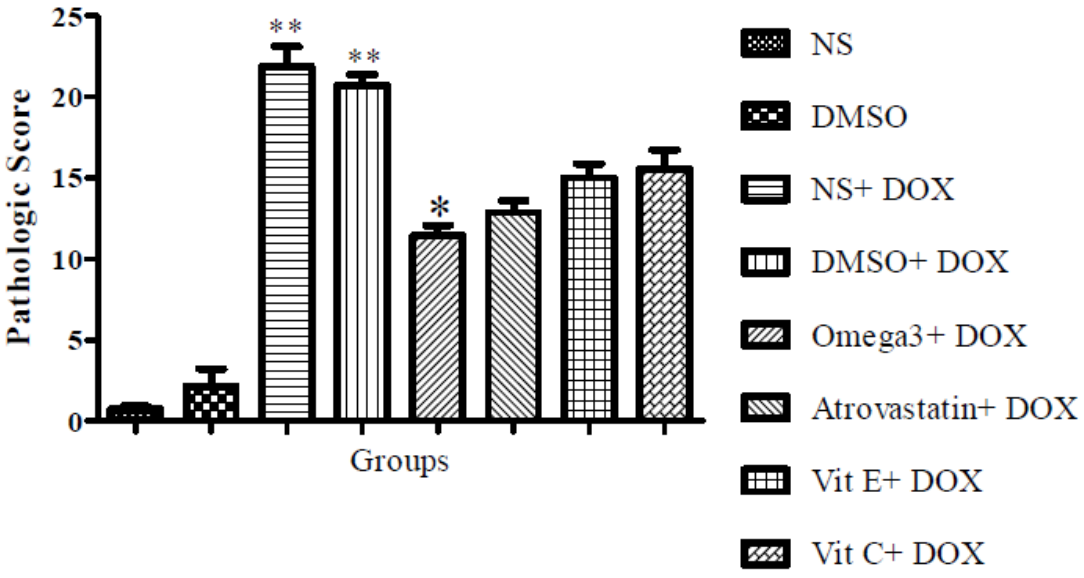

Fig.3. The mean of pathologic score in the studied groups. *significant to NS+DOX and DMSO+DOX (P<0.05). **significant to NS and DMSO (P<0.001); Agent+DOX: the groups that were taken agents before DOX injection for 14 days.

Figure 2 indicates the cardiac tissue MDA (nanomolar/g cardiac weight) in the studied groups. According to that, there was a significant difference between groups (F (7, 48) = 37.54; *P*<0.0001). Pretreatment with omega-3, atorvastatin vitamin C and E have resulted in significant decline of the cardiac MDA related to groups receiving NS or DMSO+DOX (*P*<0.0001). Meanwhile omega-3 led to less significant MDA value especially compared to atorvastatin (*P*<0.05).

Due to DOX injection, the pathologic features of heart in the groups show significant differences ($\chi^2(7)$ = 47.931; *P*<0.0001). So that it resulted in more significant the pathologic score in the groups receiving NS or DMSO related to groups taken only NS or DMSO but not DOX as shown in Figure 3 (*P*<0.001). Also, it indicated pretreatment with omega-3, atorvastatin, vitamin C and E have resulted in comparative decrease of the pathologic score related to groups receiving DOX+NS or DMSO, in which omega-3 could cause less significant score (*P*<0.05). The spearman correlation expressed the

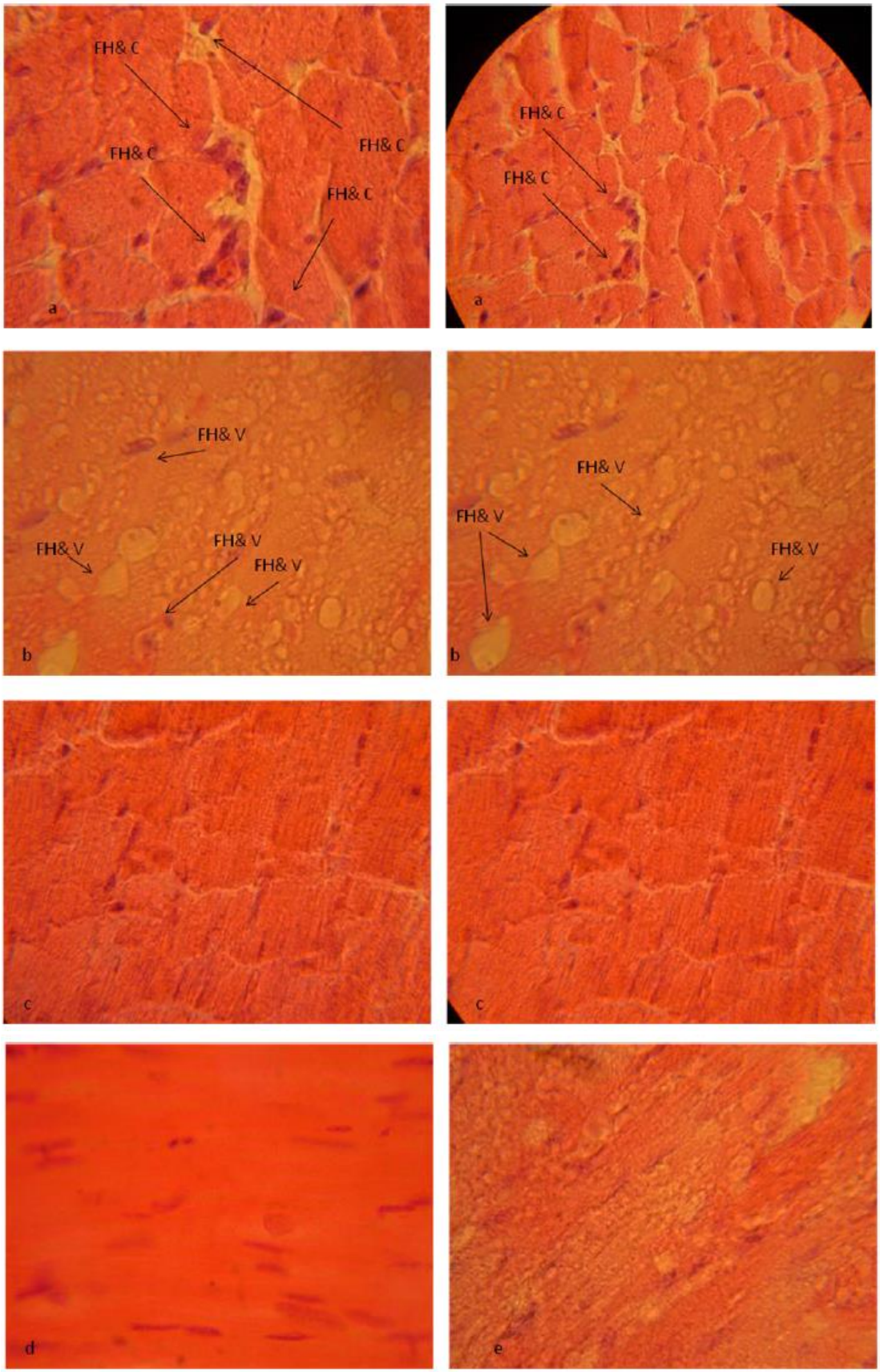

Fig. 4. The light microscope photomicrographs showing the effects of agents against histopathological alterations induced by DOX in the cardiac tissues of different experimental groups. Hematoxylin and eosin stain (H&E ×40). a: DOX+NS showing focal hemorrhage and congestion (FH& C); b: DOX+DMSO showing focal hemorrhage and vacuolization (FH& V); c: DOX+omega-3 showing a protective effect; d: normal saline group showing a no clear pathologic feature and e: DMSO group showing a no clear pathologic feature.

most correlation between the stress-oxidative index and pathologic scores for the group receiving omega-3 during both of before and after DOX administration (r= 0.9771241 for pre; r= 0.95525576 for post) (data not shown).

Finally Figure 4 illustrates the light microscope photomicrographs showing the protective effects of agents against the histopathological alterations induced by DOX in the cardiac tissues of different experimental groups.

Discussion

An increase of antioxidant capacity was observed during pre-DOX injection especially for the vitamin C and E groups so that the injection of DOX leaded to an interaction with all agents except NS in which showing rise of the level of antioxidant capacity of plasma.

There was the conflict with expected antioxidant properties of omega-3 such it exacerbated left ventricular dysfunction and decreased the ejection fraction caused by DOX (Carbone et al., 2012). But our results showed that omega-3 exerted the most protection against DOX shown by the FRAP index increase along with the cardiopathologic scores and MDA value decline compared to other tried agents. More observed efficacy of omega-3 may be related to synergism of its applied different mechanisms involved in defense pathways. Omega-3 fatty acids potentiates the efficacy of anthracyclines such DOX by enhancing the susceptibility of cell membranes to lipid peroxidation (Germain et al., 2003) by the changes in tumor fatty acid composition and enhancement of cytotoxicity of DOX to tumor cells with the protection of normal cells (Pardini, 2006) result in the anthracycline therapeutic index increase (Germain et al., 2003). The unsaturated fatty acids of omega-3 like eicosapentaenoic acid or docosahexaenoic acid reduce the side effects of anthracyclines (Hardman et al., 2002). Also, they improve the histopathologic appearances like the disorganization and loss of myocardial muscle fibers, the cytoplasmic vacuoles (Uygur et al., 2014) and decrease the mitochondrial uncoupling proteins expression (Hsu et al., 2014). Omega-3 fatty acids can attenuate MDA while increase superoxide dismutase, catalase and glutathione peroxidase activities (Changizi-Ashtiyani et al., 2012; Najafi H et al., 2017; Uygur et al., 2014; Yu et al., 2013). Alpha-linolenic acid can restore some of DOX-induced imbalance related to pro-inflammatory cytokines like nuclear factor-κB, tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) (Teng et al., 2010) or linked to apoptosis pathway such Bcl-2 and signal regulated kinase pathway like phospho-extracellular signal regulated kinases (phospho-ERKs) (Yu et al., 2013). As a result, the multiple interferences of omega-3 can explain the concurrent efficiency that consider to the FRAP index, cardio pathologic scores and MDA value in the present work.

Present results showed that vitamin E can impose a protective against DOX-induced damages. Although its efficacy to reduce the pathologic features was lower than omega-3. Vitamin E refines the extracellular collagen deposition, lipid vacuolation as well mitochondrial and dilatation (Kalender et al., 2002; Puri et al., 2005). It also decreases the levels of cardiac injury markers i.e. serum creatine phosphokinase, serum glutamic oxaloacetic transaminas, lactate dehydrogenase (Puri et al., 2005; Shah et al., 2009) as well as troponin I, IL-6 and cystatin-C (Youssef et al., 2011). The remarkable vitamin E effects on increases superoxide dismutase, glutathione peroxidase, catalase, lipid peroxidation and MDA levels (Arozal et al., 2015; Hadi et al., 2012; Kalender et al., 2002; Kalender et al., 2004; Santos et al., 2007; Tsai et al., 2010) may be involved in the significant effect on stress-oxidative parameter. Nevertheless vitamin E appears to aggravate cardiotoxicity owing to DOX overdose (Liu and Tan. 2002). It is possible that less ultimate effect of vitamin E than omega-3 is due to these contradictory interventions.

The different aspects of antioxidant activity of vitamin C have been studied. In addition to the scavenging of reactive nitrogen and oxygen species (Amara-Mokrane et al., 1996; Santos et al., 2007; Simunek et al., 2009), it can help to regenerate other antioxidants like α-tocopherol, glutathione peroxidase, urate and β-carotene from their respective radical species (Ludke et al., 2012a; Simunek et al., 2009). Then vitamin C has the potential ability to mitigate DOX-induced changes by decreasing lipid peroxide and oxidative stress (Akolkar et al., 2017; Santos et al., 2007; Simunek et al., 2009; Viswanatha Swamy et al., 2011) result in up-regulation of sodium-dependent vitamin C transporter-2 in the sarcolemma reduced

by DOX (Ludke et al., 2012b). Moreover, vitamin C corrects other adverse appearance like the dilatation of sarcoplasmic reticulum, transverse tubular system and the cytoplasmic fat droplets (Shimpo et al., 1991). Also, regarding to DOX-induced disturbances, ascorbic acid has shown the inhibition of p38 mitogen-activated protein kinase-dependent oxidative stress (Wold et al., 2005) and caspase-3 as a mediator of apoptosis (Chularojmontri et al., 2005). These reports can are consistent with our results to the most FRAP value changes by vitamin C along with vitamin E. Despite effective role of vitamin C, that is not able to suppress the DOX-induced p53 activation (Chularojmontri et al., 2005). However, it has been shown that administration of vitamin C alone had no revisory on some DOX-induced problems like the QRS or enzymes changes; even a harmful effect has been observed. Somehow that vitamin C exacerbated Dox toxicity due to an undefined cellular mechanism (Injac et al., 2009). These contradictions may be relevant to our results in which the least effect of vitamin C on the total pathologic score with even significant difference to omega-3 was observed.

The present work showed that atorvastatin could reduce the pathologic features nearly to the observed omega-3 effects. Also it can exert the most percent changing of the stress-oxidative markers like FRAP as before and after DOX injection. The noticeable effects of atorvastatin can be justified by some reports regarding its protective effects against DOX-induced cardiotoxicity. These agents exert anti-inflammatory and antioxidant effects thereby DOX-induced inflammation can be alleviated (Cappetta et al., 2017). Statins can attenuate the production of TNF-α induced by DOX and encourage the mitochondrial anti-apoptosis enzymes like SOD2 (El-Moselhy and El-Sheikh, 2014; Feleszko et al., 2000; Riad et al., 2009). Atorvastatin reduces troponin T release by cardiomyocytes (Feleszko et al., 2000) and the complications triggered by DNA damage, including the activation of p53 and stress-activated protein kinases (SAPK/JNK) that can modify small G proteins signaling such as Ras, Rho or Rac in DOX-treated (Damrot et al., 2006; Henninger and Fritz, 2017; Huelsenbeck et al., 2011; Octavia et al., 2012; Seicean et al., 2012; Yoshida et al., 2009). Statins can restore the pro-fibrotic signal transduction provoked by DOX like cytokine connective tissue growth factor, GTPases Rac1 and atrial natriuretic peptide leading to the pleiotropic, geno-protective and DNA repair effects (Henninger and Fritz, 2017; Huelsenbeck et al., 2011).

The main objective of present study was a comparison of efficiency some known agents to the reduction of DOX toxicity regarding to some aspects like the stress-oxidative and pathologic features. But just only one dose was chosen for each drug accord to previous works. Therefore, to attainment of dose-response curve and better comparison other doses should be tried. This was a limitation of our work. Also, this study need further investigations to throw more light whether intended agents can attenuate other DOX-induced markers or the cardiac injury and provide effective protection to heart tissue.

Conclusion

Our results showed vitamin E, vitamin C, omega3 and atorvastatin can exert the positive action against DOX adverse effects. However, with regard to the investigated parameters at present study like the FRAP index, histopathological scores and MDA value, omega-3 showed the most efficiency. It seems the multiple interferences of omega-3 can create better cardio protection against DOX toxicity compared to other tried agents.

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Conflict of interest

The authors have no conflict of interests to declare.

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