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Original article

Roles of some antioxidants in modulation of cardiac myopathy induced by sodium nitrite via down-regulation of mRNA expression of NF-κB, Bax, and flt-1 and suppressing DNA damage

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ABSTRACT

The underlying pathology of cardiac damage involves various molecular and signaling pathways. Therefore, this study aimed to explore the role of Quercetin (Querc), alone or in combination with Melatonin (Melat) against cardiac damage induced by sodium nitrite (Sod nit), as well as to elucidate different signaling pathways. Querc and Melat were injected intraperitoneally (i.p.), followed by induction of hypoxia in rats by using a single dose of Sod nit (60 mg/kg, s.c.). Treatment with Sod nit significantly decreased hemoglobin (Hb) levels in blood. Pretreatment of hypoxic rats with Querc and/or Melat elevated the declined Hb concentration. The forementioned antioxidants also successfully ameliorated the alteration of heat shock protein 70 (HSP-70) and markers of cardiac injury, including troponin T (Trop. T), creatine kinase-MB (CK-MB), tumor necrosis factor-α (TNF α), and C-reactive protein (CRP) in the rats serum. Furthermore, RT-PCR revealed that these antioxidants successfully modulated mRNA expression of NF-κB, Bax, Bcl-2, and flt-1. They also regulated vascular endothelial growth factor (VEGF), the apoptosis marker caspase 3, and oxidative DNA damage in cardiac tissue, compared to Sod nit-intoxicated rats. The present biochemical results are reinforced by histopathological examination. In Conclusion: The results reflected that treatment with Querc in combination with Melat was most effective in improving Sod nit-toxicity induced cardiac damage, thus confirming the promising role of this combination as an effective treatment for cardiac damage induced by other cardio-toxic agents.

1. Introduction

Sodium nitrite (Sod nit) is a widely used as a medication for different human and animal diseases. It acts as a vasodilator, a bronchodilator, an intestinal relaxant, and an antidote for cyanide poisoning (Kroupova et al., 2005). However, overdose of Sod nit could be life threatening (Gonchar et al., 2006). The interaction between Sod nit and red blood cells (RBC) oxidizes Fe^{+2} to Fe^{+3}. This reaction results in formation of methemoglobin causing loss of the capability of RBC to carry O_2, thus ending in hypoxia (Fraser and Mays, 1986).

Al-Gayyar et al. (2014) reported that Sod nit administration causes dysregulation of inflammation, hypoxia, ischemia, oxidative stress, and impaired energy metabolism, resulting in organ damage. In many tissues, hypoxia induces expression of vascular endothelial growth factor (VEGF) mRNA by activating hypoxia inducible factor 1-α (HIF-1α), which is a major regulator of VEGF (Clerici and Planes, 2009). VEGF induces the expression of anti-apoptotic proteins in human endothelial cells, thus promoting the survival of these cells. VEGF mRNA transcripts and proteins

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are also expressed by other cell types, such as hepatocytes (Mochida et al., 1996). VEGF has two primary receptors: VEGFR1 (flt-1) and VEGFR2.

Heat shock proteins (HSPs) induced by stress play an important role in defense against cellular injury (Barral et al., 2004). Caspases initiate cell death programming caspase-3(CASP-3) acts as an initiator of the intrinsic pathway (Riedl and Shi, 2004).

Ghosh et al. (2014) reported that many conditions, including DNA damage, are mediated by oxidative stress. Bcl-2 family proteins, including Bcl-2 and Bax, play an important role in the regulation of apoptosis, and Bax/Bcl-2 ratio can influence the susceptibility of cells to apoptosis (Yao et al., 2012).

Flavonoids play vital biological effects; they act as antioxidants and scavenge free radicals (Gautam et al., 2007). Quercetin (2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one; Querc) and other flavonoids show anti-prostanoid and anti-inflammatory responses, protect low-density lipoprotein from oxidation, prevent platelet aggregation, and promote relaxation of cardiovascular smooth muscles. Querc protects liver cells from carbon tetrachloride toxicity and cyclosporin-induced nephrotoxicity (Pavanato et al., 2003).

Melatonin (N-acetyl-5-methoxytryptamine; Melat) is a neuro hormone produced by the pineal gland, which is involved in the regulation of natural circadian rhythm (Lundmark et al., 2006). It is used as a therapeutic agent to ameliorate molecular and organ/tissue damage and serves as a free radical scavenger and excitotoxicity mitigator (Ramos et al., 2017). Furthermore, it prevents cancer by maintaining DNA integrity (Gitto et al., 2012).

The aim of the present study was to evaluate protective effects of Querc and Melat on cardiac myopathy induced by Sod nit, as well as to elucidate different signaling pathways.

2. Material and methods

2.1. Chemicals

All chemicals used in the study were products of Sigma and Merck companies of high analytical grade.

2.2. Experimental animals

Fifty Wistar adult male albino rats weighing 170–200 g were obtained from the Experimental Animal House, Faculty of Pharmacy, King Saud University, Saudi Arabia. Procedures outlined by the Experimental Animal Ethics Committee were followed. Animals were maintained under standard conditions of temperature and humidity. Rats were fed with standard rat pellet chow with free access to tap water ad libitum for 1 week before the experiment to allow acclimatization. Rats were divided into five groups of ten rats each: Group 1, control; Group 2, Sod nit-treated animals (60 mg/kg; Cigerci et al., 2009); Group 3, Sod nit-treated animals pre-injected with Querc (200 mg/kg, i.p.; Gautam et al., 2007); Group 4, Sod nit-treated animals pre-injected with Melat (200 mg/kg, i.p.; Rao et al., 2000); Group 5, Sod nit-treated animals intraperitoneally injected with a combination of Querc (200 mg/kg) and Melat (200 mg/kg).

A single dose of Sod nit was treated (60 mg/kg) subcutaneously. Querc and Melat were administered 24 h and 1 h before Sod nit injection. One hour after Sod nit injection, the rats were killed, and blood samples were collected for determining ***hemoglobin (Hb)*** and another portion for serum separation via centrifugation at 3000*g* for 10 min, and the supernatants and the serum were stored at −80°C and used for further biochemical analysis. The heart samples were collected, washed, minced, and homogenized in phosphate buffer to yield 20% homogenates. These were centrifuged at 4042*g*. Four hearts from each group were maintained in 4% formalin for histopathological examination.

2.3. Biochemical blood analysis

2.3.1. Determination of hemoglobin (Hb)

Hb was determined colorimetrically using Drabkin's reagent, according to the method described by Kjeldsberg (1993).

2.3.2. Biochemical serum analysis

2.3.2.1 Determination of heat chock protein-70 (HSP-70), troponin T (Trop. T), creatine kinase-MB (CK-MB), tumor necrosis factor-α (TNFα) and C-reactive protein (CRP). For the quantitative determination of HSP-70, a sandwich rat HSP-70 ELISA Kit (Kamiya Biomedical, Washington) was used. Troponin T (Trop. T) concentration was determined using a Siemens Dimension Xpand Plus instrument (IL, USA). CK-MB was estimated spectrophotometrically using a standard enzyme kit supplied by Spinreact, S.A.-Spain (Cat. No. 1001055). TNF α was measured using a high sensitive rat (ELISA) kit (IBL International GmbH, Flughafenstr, Hamburg, Germany) following instructions of the manufacturer. CRP was estimated using immunonephelometric assay (Dade Behring N Latex High Sensitivity CRPTM mono assay) on a Behring Nephelometer II analyzer.

2.4. Biochemical cardiac tissue analysis

2.4.1. Assay of caspase 3 activity

Caspase 3 activity was assayed according to the method described by Vaculova and Zhivotovsky (2008).

2.4.2. Vascular endothelial growth factor (VEGF)

The level of VEGF was determined using kits (ELISA; R&D Systems, UK) in accordance with the manufacturer's instructions.

2.5. Quantitative Real-Time Polymerase Chain Reaction (Qrt-Pcr) for analysis of hepatic NF-κB, Bax, Bcl-2 and flt-1 mRNA expression

2.5.1. Total RNA extraction

Total RNA was isolated from cardiac tissue homogenates using RNeasy Purification Reagent (Qiagen, Valencia, CA), according to manufacturer's instructions. RNA quality was confirmed by gel electrophoresis on a 1% agarose gel stained with ethidium bromide.

2.5.2. cDNA synthesis and qRT-PCR

First-strand cDNA was synthesized from 4 μg of total RNA using an Oligo(dT) 12–18 primer and Superscript™ II RNase Reverse Transcriptase.

Equal amounts of RNA (2 μg) were reverse transcribed into cDNA using Superscript Choice systems (Life Technologies, Breda, Netherlands), according to the manufacturer's instructions. To assess the mRNA expression of NF-κB, Bax, Bcl-2, and flt-1, quantitative real-time PCR was performed using SYBR green PCR Master mix (Applied Biosystems, CA, USA), as described by the manufacturer. Briefly, in a 25 μL reaction volume, the following components were added: 5 μL of cDNA, 12.5 μL of 2× SYBR green Master Mix, 200 ng of each primer, and 5 μL RNase-free water. The sequences of primers are given in Table 1. The relative expression was calculated using $2^{-\Delta\Delta CT}$ formula (Livak and Schmittgen, 2001).

2.6. Comet assay

It is single cell gel electrophoresis, described by Singh et al. (1988),

Table 1
Primer sequences used for RT-PCR.

Gene name	Primer sequence	Primer size (bp)
Refer-actin	Forward 5′ GAGACCTTCAACACCCCAGC 3′ Reverse 5′ ATGTCACGCACGATTTCCC 3′	263
NF-κB	Forward 5′ CATGAAGAGAAGACACTGACCATGGAAA3′ Reverse 5′ TGGATAGAGGCTAAGTGT AGACACG 3′	329
Bax	Forward 5′ GTTGCCCTCTTCTACTTTG 3′ Reverse 5′ AGCCACCCTGGTCTTG 3′	194
Bcl-2	Forward 5′ CGGGAGAACAGGGTATGA 3′ Reverse 5′ CAGGCTGGAAGGAGAAGAT 3′	224
Flt-1	Forward 5′-CAAGGGACTCTACACTTGTC-3′ Reverse 5′-CCGAATAGCGAGCAGATTTC-3′	267

F: Forward primer sequence R: Reverse primer sequence.

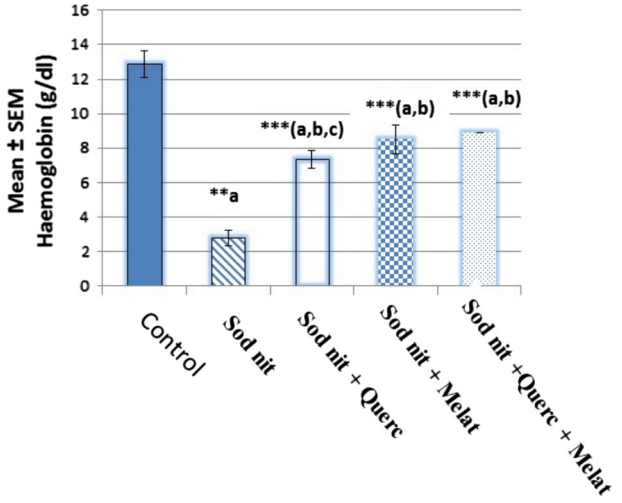

Fig. 1. Effect of Melat and/or Querc on blood Hb in control and different treated groups. Data are expressed as means ± SEM (n = 10). $^{***}p \leq .001$, $^{*}p \leq .01$ were considered significant. a: compared with the control group; b: compared with hypoxia group; c: compared with the Sod nit and combination treated group.

2.7. Histopathological examination

Small pieces of the heart were fixed in 4% formalin, embedded into paraffin, followed by staining of sections with hematoxylin-eosin (Smith and Bruton, 1978).

2.8. Statistical analysis

Data were statistically analyzed by comparing the values of different groups with the values of controls. Results are expressed as mean ± SEM. Significant differences among the values were analyzed using ANOVA test, followed by Bonferroni's test post-ANOVA. Limits of significance are listed as $p \leq .001$, $p \leq .01$ and $p \leq .05$.

3. Result

Fig. 1 shows that Sod nit-treatment significantly reduced Hb concentration in blood, as compared to the control group ($p \leq .01$). Pre-administration of Querc and/or Melat caused significant amelioration of Hb concentration, as compared to both control and hypoxic groups ($p \leq .001$). Administration of Querc and Melat combination showed the maximum effect.

The level of HSP-70 was increased significantly in Sodium nit intoxicated rats, as compared with the control group (Table 2). Pre-administration with Querc and/or Melat markedly reduced this increase. Serum cardiac biomarkers, i.e., CK-MB and Trop. T in the untreated and various experimental groups intoxicated with Sod nit are shown in Table 2. The damage induced by Sod nit was markedly increased in these biomarkers, compared with control animals ($p \leq .001$), and the consumption of Querc and/or Melat significantly decreased the deviation in these markers, compared with Sod nit intoxicated group.

The levels of some immunological pro-inflammatory biomarkers, including TNFα and CRP, in cardiac tissue intoxicated with Sod nit were much higher, compared with controls ($p \leq .001$). Pre-treatment with Querc and/or Melat noticeably inhibited the induced inflammatory mediators, compared with intoxicated animals.

Sod nit treatment significantly elevated the levels of both VEGF (angiogenic factor) and CASP-3 ($p \leq .001$). However, pre-treatment with the aforementioned antioxidants ameliorated this elevation (Fig. 2).

The administration of Sod nit induced significant elevation in NF-κB mRNA expression than that in the control group ($p \leq .001$), while pre-treatment of the rats with Querc, alone or in combination with Melat, caused significant reduction of these levels, compared with Sod nit-treated rats ($p \leq .001$) (Fig. 3A).

Fig. 3B shows that the proapoptotic protein (Bax) mRNA expression level was increased significantly after Sod nit administration, compared with control group ($p \leq .001$). These elevated levels reduced significantly with co-treatment with Querc and/or Melat, compared with Sod nit-treated group ($p \leq .001$). The anti-apoptotic protein Bcl-2 mRNA expression levels reduced significantly in the Sod nit intoxicated rats, compared with control group ($p \leq .001$). Treatment of Sod nit group with the aforementioned antioxidants ameliorated these levels, as compared with Sod nit-treated groups. Co-administration of combination of Querc and Melat ameliorated Bcl-2 mRNA expression levels. This amelioration was significant, compared with that observed in Sod nit treated group ($p \leq .001$) (Fig. 3C).

Table 2
Effect of Melat and/or Querc on HSP 70, Trop T, CK-MB, TNF α and CRP in the serum of control and different treated groups.

Groups/parameter	Mean + SE HSP70 ng/ml	Mean + SE Trop. T Pg/ml	Mean + SE CK-MB U/L	Mean + SE TNFα Pg/ml	Mean + SE CRP ng/ml
Control	5.58 ± 0.11	34.6 ± 2.2	26.49 ± 2.2	208.8 ± 8.6	9.1 ± 0.38
Sod nit	8.05 ± 0.33^{***a}	63.33 ± 2.8^{***a}	44.8 ± 1.3^{***a}	543.7 ± 15.9^{***a}	18.4 ± 0.76^{***a}
Sod nit & Querc	7.1 ± 0.28^{*a}	43.97 ± 1.3^{**b}	38.75 ± 1.7^{**a}	412.3 ± 49.7$^{**a/*b}$	14.8 ± 1.01$^{**a/*b}$
Sod nit & Melat	6.4 ± 0.23^{*b}	48.78 ± 3.7$^{*a/*b}$	33.55 ± 2.2^{*b}	333.1 ± 9.9 $^{*a/***b}$	14.7 ± 0.7$^{**a/*b}$
Sod nit & Querc & Melat	6.84 ± 0.28	41.72 ± 1.1^{***b}	32.71 ± 1.6^{**b}	430.5 ± 10.4^{***a}	11.6 ± 0.46^{***b}

Data are expressed as means ± SEM (n = 10). $^{***}P \leq .001$, $^{**}P \leq .01$ and $^{*}P \leq .05$ were considered significant.
[a] Compared with the control group.
[b] Compared with Sod nit-treated group.

Flt-1 mRNA expression level in Sod nit treated group were significantly higher than those in the control group ($p \leq .001$). Treatment of rats with Querc and Melat caused significant reduction of these levels, as compared with those observed in Sod nit treated rats ($p \leq .001$) (Fig. 3D).

The effect of the post treatment with Sod nit on DNA in rat hearts is shown in Fig. 4. Significant increase in the tail length and DNA% (tail DNA content) were shown in the cardiac tissues of rats intoxicated with Sod nit. Pretreatment with Querc and Melat to Sod nit-intoxicated rats significantly protected their

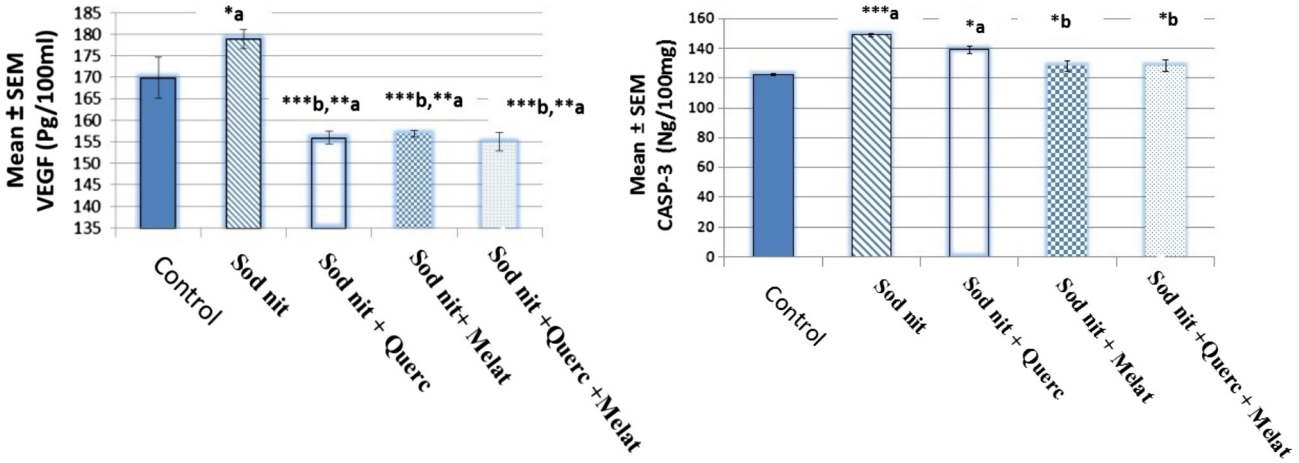

Fig. 2. Effect of Querc and/or Melat on the levels of inflammatory and angiogenic markers in hypoxic cardiac tissues of rats. $^{***}p \leq .001$, $^{**}p \leq .01$; $^{*}p \leq .05$ were considered significant; Data are expressed as mean ± SEM; n = 10. a: Significantly different from control group. b: Significantly different from the hypoxic-treated group. c: Significantly different from Sod nit and combination-treated group.

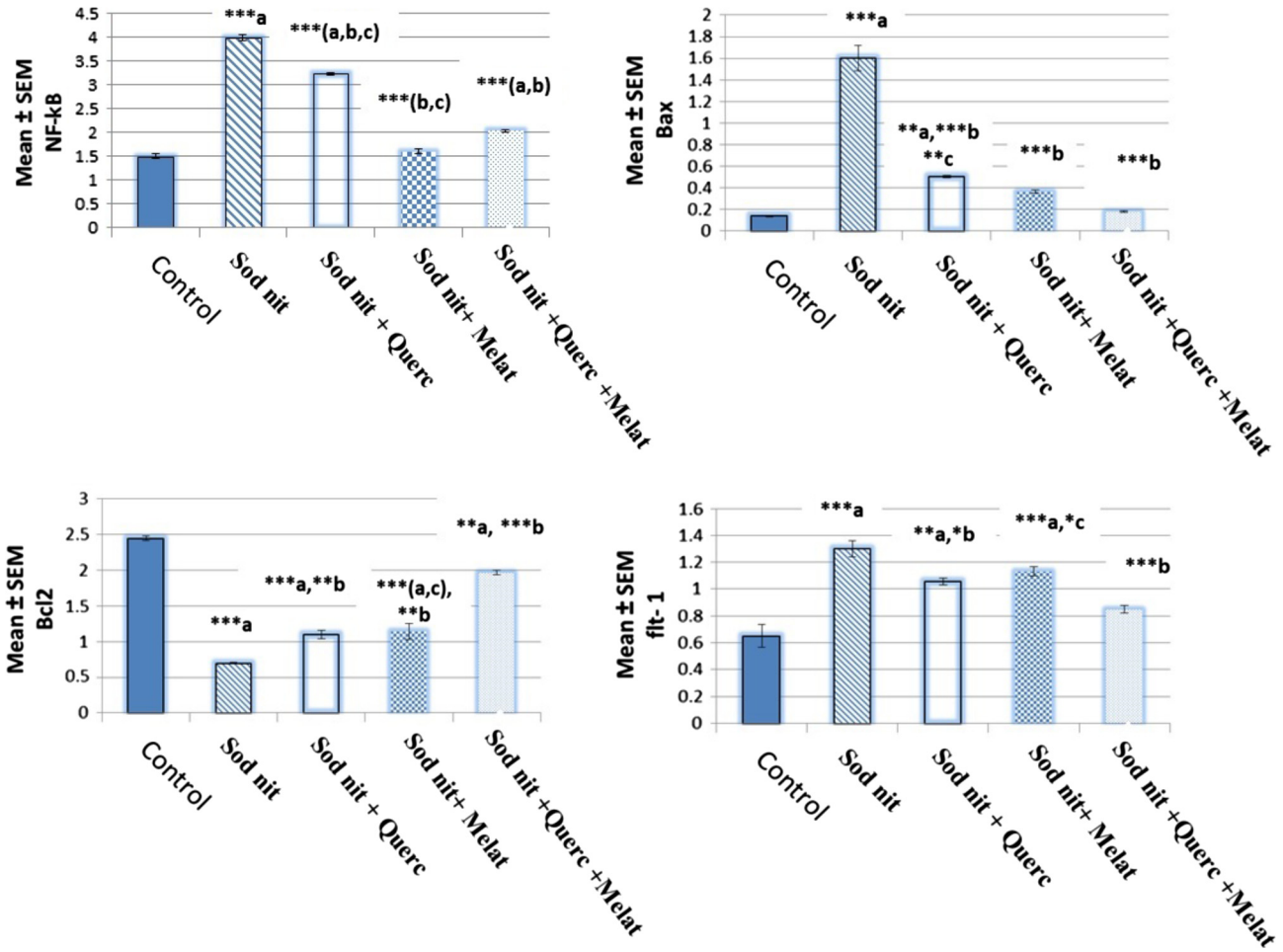

Fig. 3. NF-κB, Bax, Bcl2 and flt-1 mRNA expression in the heart of control and different treated groups. $^{***}p \leq .001$, $^{**}p \leq .01$ and $^{*}p \leq .05$ were considered significant; Data are expressed as mean ± SEM; n = 10. a: Significantly different from control group. b: Significantly different from the Sod nit-treated group. c: Significantly different from the Sod nit and combination-treated group.

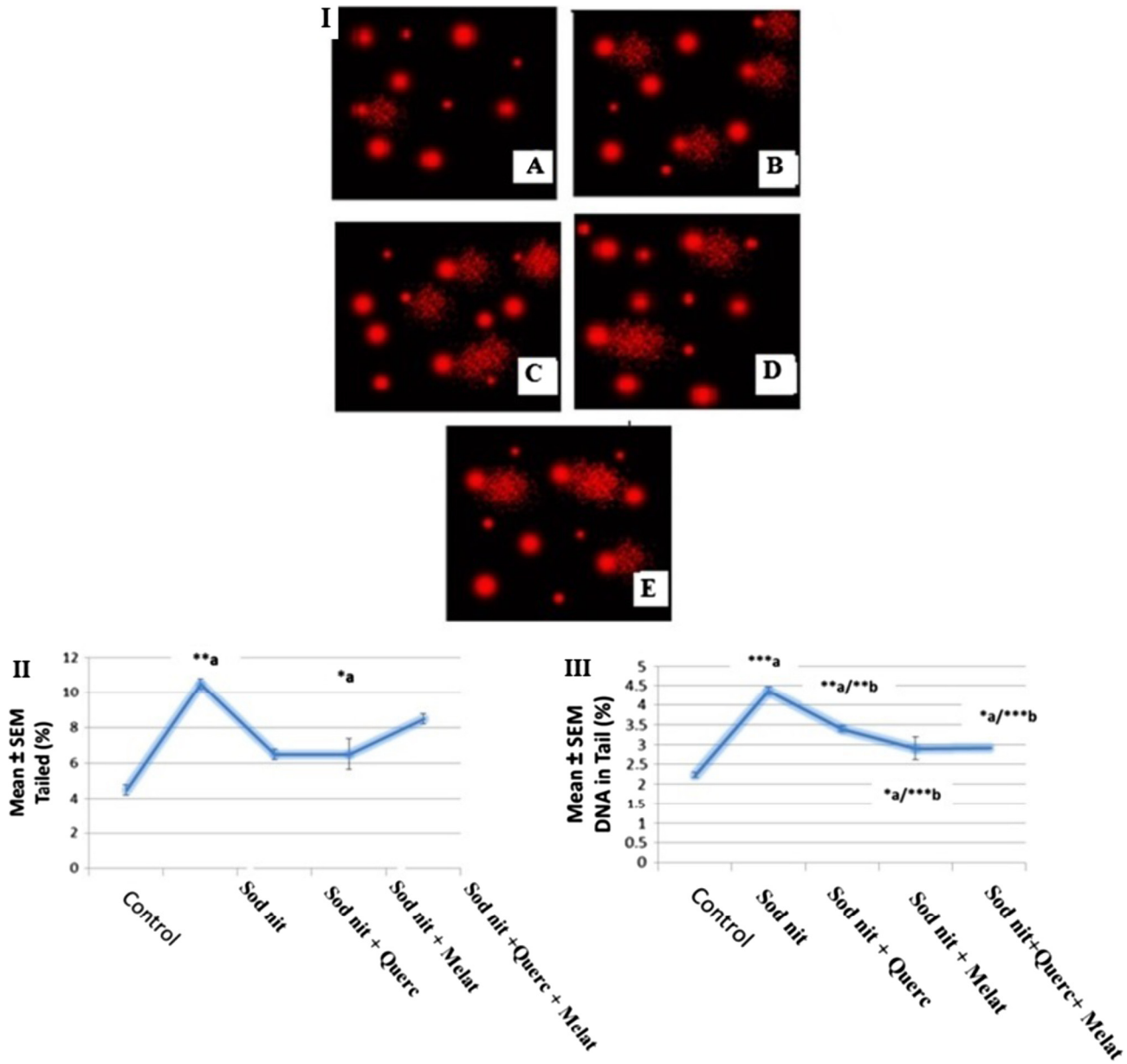

Fig. 4. (I) DNA damage in the cardiac tissues of Sod nit intoxicated rats and the effect of Querc or/and Melat treatment on the level of DNA damage. Comet assay showing the degree of DNA damage in the cardiac tissues of (a) normal control group, (b) group intoxicated with the Sod nit, (c) intoxicated group treated with Querc, (d) intoxicated group treated with Melat (e) intoxicated group treated with Querc and Melat. (II) Effect of Querc and/or Melat on Tail – DNA in Sod nit rats. (III) Effect of Querc and/or Melat on tail length in hypoxic rats. $^{***}p \leq .001$, $^{**}p \leq .01$ and $^{*}p \leq .05$ were considered significant; Data are expressed as mean ± SEM; n = 10. a: Significantly different from control group. b: Significantly different from the hypoxic-treated group. c: Significantly different from the Sod nit and combination-treated group.

hearts from DNA damage, as indicated by a decrease in the above markers of DNA damage, compared with those in intoxicated untreated rats ($p \leq .001$).

Histopathological examination of the heart tissues of rats stained with Hematoxylin and Eosin showed that hypoxia induced a marked degeneration of myocardial cells and widening of the endomysium due to vascular congestion and cellular infiltration, while treatment with Melat, Querc, and their combination markedly improved the myocardial cells and markedly decreased of the cellular infiltration, especially in rats treated with a combination of Melat and Querc (Fig. 5).

4. Discussion

Sod nit forms peroxynitrite ($ONOO^-$), which has strong oxidative capacity causing cellular oxidative DNA destruction (Virag et al., 1999), via interaction between produced NO and superoxides ($O_2^{\cdot-}$) (Jay-Gerin and Ferradini, 2000).

The results of the present study revealed that Sod nit significantly decreased Hb concentration; this is in coincide with that of Al-Gayyar et al. (2014) who reported that hypoxia induced by Sod nit is accompanied by oxidative stress, inflammation, and methemoglobinemia.

Sod nit induced cardiotoxicity as indicated by elevated cardiac biomarkers, Trop T and CPK-MB, in serum. Troponins control the calcium-mediated interaction between actin and myosin (Adamcova et al., 1997).

The present study showed increased levels of HSP-70, HIF-1α, TNFα, and CRP, which may result in cardiac damage, in Sod nit-intoxicated rats. HSPs, which are proteins misfolded by oxidative stress, are controlled by the HIF-1α pathway in hypoxia (Baird et al., 2006). The rise in the inflammatory cytokines by Sod nit

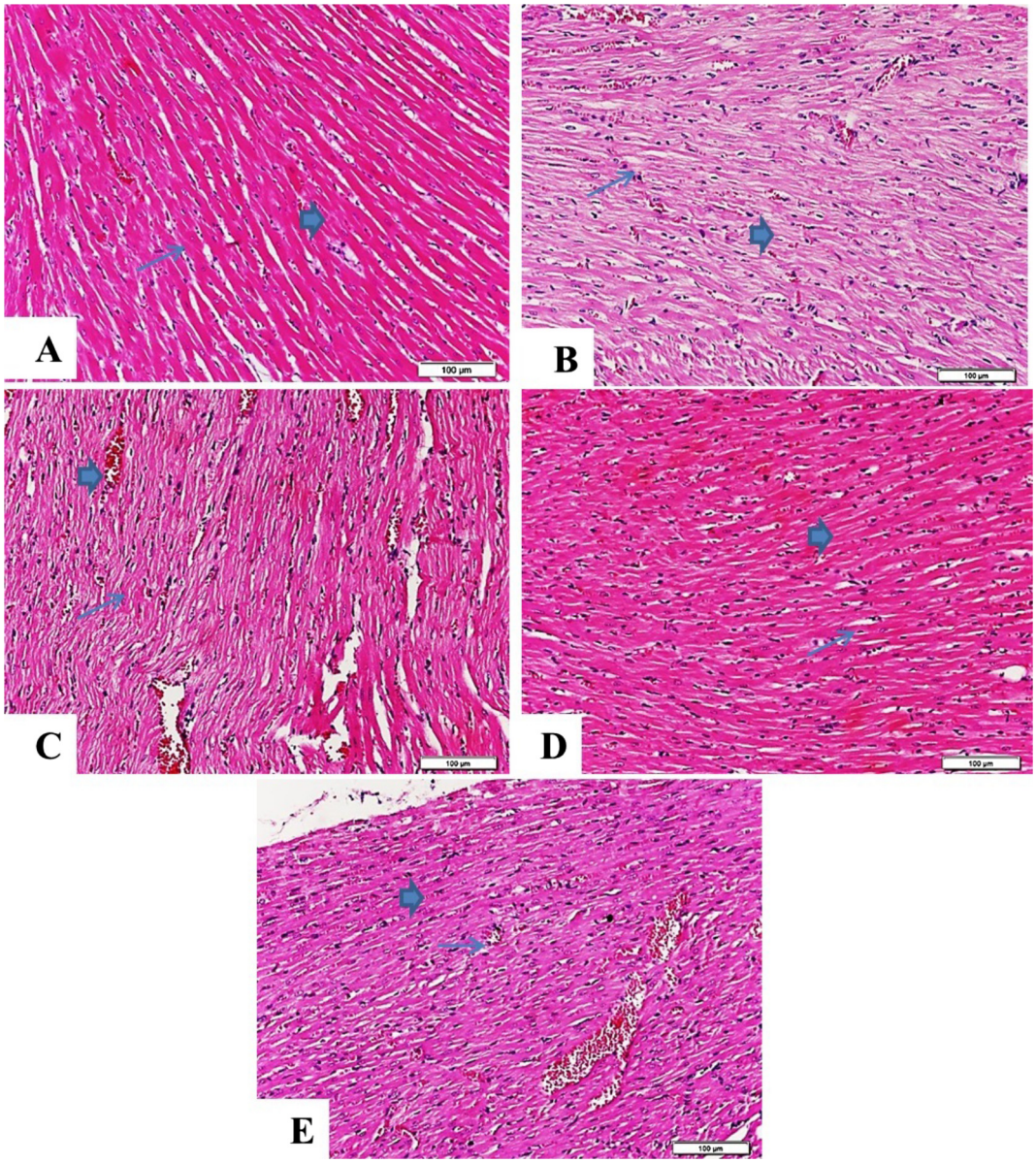

Fig. 5. Light photomicrographs of heart from rat stained with Hematoxylin and Eosin (Scale bar: 100 µm), in which (A) represent normal myocardial cells (arrowhead) and endomysium (arrow). (B) Section of heart from rat exposed to hypoxia showing marked degeneration of myocardial cells (arrowhead) and widening of the endomysium due to vascular congestion and cellular infiltration (arrow). (C, D and E) are sections of heart from rat exposed to hypoxia and received Melat, Querc and combination of both of them respectively, showing marked improvement of the myocardial cells and marked decrease of the cellular infiltration specially in rats received combination of Melat and Querc (E).

may be correlated to activation of NF-κB that is responsible for producing inflammatory cytokines (Li et al., 2012). These biomarkers play a vital role in cardiac infarction and damage induced by Sod nit toxicity. This is corroborated by findings of previous studies reporting that TNFα is involved in the pathophysiology of cardiac muscles failure (Irwin et al., 1999).

Moreover, Sod nit significantly elevated Bax, NF-kB, and flt-1 levels and suppressed Bcl2 mRNA expression in rat's heart. Administration of Querc and/or Melat regulated the expression of these markers.

Depletion in ATP levels induced the translocation of mitochondrial cytosolic Bax to outer membrane of the mitochondria, leading to mitochondrial dysfunction and enlargement and movement of cytochrome *c* to cytosol (Malhi et al., 2006). Bcl2 prevents cell death. On the contrary, Bax initiates cell death signal (Green and Reed, 1998). The present work confirmed a significant decrease in Bcl2 expression, complemented by increase in Bax levels after Sod nit treatment, compared with those observed in normal animals. Querc and/or Melat treatment increased levels of the anti-apoptotic Bcl2 and reduced the pro-apoptotic protein Bax levels, supporting their anti-apoptotic role ($p \leq .001$).

The present study showed a marked increase in the angiogenic index, VEGF in the cardiac tissues of Sod nit treated rats. This is in accordance with findings of Pham et al. (2002) who showed that hypoxia is a strong inducer of VEGF expression in many tissues. This finding may indicate that extensive formation of TNF-α and VEGF in Sod nit-rats could initiate cardiac dysfunction. Pre-administration of the examined materials, alone or in a combination, down-regulated the increase in VEGF expression in the cardiac tissues of Sod nit treated animals, suggesting their potential anti-angiogenic action.

Melat shows an anti-tumor effect through multiple mechanisms, but these are not yet fully established. It suppressed expression of VEGF gene by inhibition of accumulation of HIF α under hypoxic environment (Cui et al., 2012).

Molkentin (2001) documented that stimulation of caspases and DNA fragmentation enzymes results from depletion of energy in cardiac tissues.

Non-DNA mediated process or apoptosis induces DNA damage resulting in cell death (Tice and Strauss, 1995). As a result of DNA breaking, parts of DNA move to the comet tail, and in extreme cases (the apoptotic cell), both the head and the tail separate. Incidence of breaks is indicated by tail length and percentage of total DNA in the tail (ColLins et al., 1996).

Some studies demonstrated that NO-derived reactive nitrogen species induce disease potential, including DNA and tissue damage, leading to increased mutation rates, genome instability, apoptosis, and associated tissue regeneration, encompassing a proliferative response of cells (Sawa and Ohshima, 2006). In addition, DNA damage and apoptosis in cells may result from exposure to both hyperoxia and hypoxia (Poon et al., 2007).

In this study, administration of Querc and/or Melat to Sod nit-intoxicated animals successfully mitigated the alteration in the above markers of DNA damage in their cardiac tissue. Nair et al. (2002) showed that Querc has antitumor, anti-inflammatory, anti-allergic, and antiviral activities, and Melat mitigates the increase in brain stress markers leading to lipid peroxidation and DNA damage (Szárszoi et al., 2001).

The destructive effect of Sod nit on cardiac tissue was detected in histomorphological images showing degeneration.

In conclusion, this study demonstrated that administration of Querc and/or Melat ameliorated the cardiac injury induced by inflammatory mediators, as well as oxidative DNA damage and apoptosis caused by toxic effects of Sod nit. These results support potential application of this combination in cardiomyopathy treatment.

Acknowledgments

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