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Original article

CoQ10 augments candesartan protective effect against tourniquet-induced hind limb ischemia-reperfusion: Involvement of non-classical RAS and ROS pathways

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ABSTRACT

Tourniquet is a well-established model of hind limb ischemia–reperfusion (HLI/R) in rats. Nevertheless, measures should be taken to alleviate the expected injury from ischemia/ reperfusion (I/R). In the present study, 30 adult male Sprague-Dawley rats were randomly divided into 5 groups (n = 6): control, HLI/R, HLI/R given candesartan (1 mg/kg, P.O); HLI/R given Coenzyme Q10 (CoQ10) (10 mg/kg, P.O); HLI/R given candesartan (0.5 mg/kg) and CoQ10 (5 mg/kg). The drugs were administered for 7 days starting one hour after reperfusion. Candesartan and CoQ10 as well as their combination suppressed gastrocnemius content of angiotensin II while they raised angiotensin-converting enzyme 2 (ACE2) activity, angiotensin (1–7) expression, and Mas receptor mRNA level. Consequently, candesartan and/or CoQ10 reversed the oxidative stress and inflammatory changes that occurred following HLI/R as demonstrated by the rise of SOD activity and the decline of MDA, TNF-α, and IL-6 skeletal muscle content. Additionally, candesartan and/or CoQ10 diminished gastrocnemius active caspase-3 level and phospho-p38 MAPK protein expression. Our study proved that CoQ10 enhanced the beneficial effect of candesartan in a model of tourniquet-induced HLI/R by affecting classical and non-classical renin-angiotensin system (RAS) pathway. To our knowledge, this is the first study showing the impact of CoQ10 on skeletal muscle RAS in rats.

1. Introduction

The application of a tourniquet is considered a common method of hemostasis to prevent trauma fatalities in both battlefield and civilian situations (Doyle and Taillac, 2008, Lee et al., 2007). However, tourniquet presence results in ischemia that is followed by reperfusion upon its removal leading to I/R injury. Reactive oxygen species (ROS) are formed due to poor tissue perfusion during the ischemic phase. When reperfusion follows, an inflammatory response is initiated which leads to an aggravation of muscle damage and apoptosis (Granger and Kvietys, 2015). Similarly, peripheral nerves (e.g. sciatic nerve) were found to be extremely prone to I/R insult (Iida et al., 2007).

Previous studies have proved the presence of classical (ACE/ Ang II/ AT1 receptor) and non-classical (ACE 2/ Ang (1–7)/ Mas-1 receptor) RAS pathways in skeletal muscle with counteracting effects to each other (Cabello-Verrugio et al., 2015, Fernandes et al., 2010). Ang II exerts well-known pro-oxidant and pro-inflammatory actions through the activation of AT1 receptors, leading to mitochondrial dysfunction and upregulation of pro-inflammatory genes (Kinugawa, 2017, Passos-Silva et al., 2015). On the other hand, Ang (1–7) has shown favorable effects against various skeletal muscle abnormalities through its action on Mas receptors (Cabello-Verrugio et al., 2015, Cisternas et al., 2015). Hence, blocking of Ang II action or enhancement of Ang 1–7 effect could be beneficial in treating muscle disorders.

ARBs have many cardiovascular and pleiotropic effects making them widely used in clinical practice (Chrysant and Chrysant, 2006). Candesartan, a potent ARB with a long duration of action,

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has shown to be effective in several models of ischemia–reperfusion (Culman et al., 2017, Sheik Uduman et al., 2016, Takagi et al., 2006). Interestingly, candesartan was found to exert a direct modulatory effect on the ACE 2/ Ang (1–7)/ Mas-1 receptor axis in addition to its well-known AT1 receptor blocking property (Arumugam et al., 2012, Pernomian et al., 2015).

Gastrocnemius is a glycolytic muscle characterized by having fewer mitochondria and antioxidant protection than oxidative muscles; thus it is more vulnerable to I/R injury. Upkeeping of mitochondrial and antioxidant function could afford a better defense to the muscle exposed to traumatic conditions (Charles et al., 2017).

CoQ10 is a nutraceutical with muscle supporting properties due to its established mitochondrial antioxidant capacity. Indeed, the helpful effects of CoQ10 in myopathies have been reported either experimentally or clinically (Tran et al., 2012, Woodman et al., 2016). However, to our knowledge, the impact of CoQ10 administration on the RAS pathway has not been elucidated yet.

Therefore, it was interesting for us to investigate the possible involvement of the RAS pathway in the effects exerted by CoQ10 in the skeletal muscle and elucidate its possible enhancement to candesartan action on tourniquet-induced I/R injury in rats.

2. Materials and methods

2.1. Animals

Thirty adult male Sprague–Dawley rats aged 8 weeks (200–220 g) were housed under a controlled temperature and humidity atmosphere and allowed to access pelleted food and drinking water *ad libitum*. The animal experiments described later were approved by the Ethics Committee, Faculty of Pharmacy, Ahram Canadian University, Egypt (approval number: 1/9/2020/9).

2.2. Drugs and chemicals

Candesartan and CoQ10 were supplied by Astra Zeneca and the Arab Company for Pharmaceuticals & Medicinal Plants (Cairo, Egypt), respectively. All the chemicals were of analytical grade and purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

2.3. Induction of hind limb ischemia-reperfusion and animals treatment

Rats were anesthetized using thiopental (85 mg/kg, i.p). The animals were kept on heating pads to maintain body temperature at 37° C.

Unilateral left hind limb ischemia was achieved by positioning a tourniquet consisting of an orthodontic rubber band at the hip joint (Crawford et al., 2007) for 90 min (Erkut et al., 2016). Reperfusion was initiated by removing the rubber bands. Control rats were not subjected to tourniquet application.

The animals were randomly divided into five groups (n = 6) as follows: Control group, HLI/R rats, HLI/R rats treated with candesartan (1 mg/kg) (Matsuo et al., 2002) for 7 days starting one hour after reperfusion, HLI/R rats treated with CoQ10 (10 mg/kg) (Kalayci et al., 2011) for 7 days starting one hour after reperfusion, HLI/R rats treated with a combination of candesartan (0.5 mg/kg) and CoQ10 (5 mg/kg) for 7 days starting one hour after reperfusion.

Both candesartan and CoQ10 were suspended in 10% tween 80 solution and administered orally by gavage. The same vehicle was given to the control group.

On the eighth day of the experiment, rats were sacrificed. Gastrocnemius muscles of the left limbs were harvested and washed with ice-cold normal saline. A portion of gastrocnemius muscles was homogenized in phosphate buffer saline (0.1 M PBS, pH 7.4) and centrifuged at 10000 rpm for 30 min at 4 °C and supernatants were stored at − 80 °C till biochemical parameters were measured. The second and third portions were put at 4 °C in RIPA buffer for Western blotting analysis and TRIzol solution for real-time PCR assay, respectively. The last portion of gastrocnemius muscles was fixed in 10% neutral buffered formalin solution, dehydrated, and impregnated in Paraplast medium for histopathological examination and immunohistochemistry experiment.

2.4. Biochemical parameters

2.4.1. Evaluation of ACE2 activity, angiotensin II and angiotensin (1–7) levels

ACE2 activity was determined using an enzyme-linked immunosorbent assay (Cusabio Technology, USA, Cat No. CSB-E14308r) according to the manufacturer's instructions. Angiotensin II and angiotensin (1–7) contents were determined using quantitative sandwich enzyme immunoassay (Cusabio Technology, USA, Cat. No. CSB-E04494r and CSB-E14241r, respectively) according to the supplier's protocol.

2.4.2. Determination of inflammatory biomarkers

TNF-α and IL-6 levels were determined by ELISA kits (Cat. No. SEA133Ra and SEA079Ra, respectively) using sandwich enzyme immunoassay principle (Cloud-Clone Corp., Houston, TX) and following the manufacturer's instructions.

2.4.3. Measurement of oxidative stress parameters

MDA content, as well as SOD activity, were determined using colorimetric methods as indicated by Biodiagnostic kits, Egypt (Cat. No. MD 25 29 and SD 25 21, respectively).

For determination of MDA content, tissue homogenate supernatants were mixed with chromogen containing thiobarbituric acid (25 mmol/L) and heated in a boiling water bath for 30 min. The absorbance of the samples was read at 534 nm according to the manufacturer's instructions.

SOD activity was determined in supernatants after the addition of a working reagent containing nitroblue tetrazolium dye (1 mM/L) and NADH (1 mM/L). The increase in absorbance at 560 nm was measured for 5 min after the addition of phenazine methosulphate (0.1 mM/L) according to the kit instructions.

2.4.4. Determination of active caspase-3

Active caspase-3 was determined by immunohistochemistry. Deparaffinized 5 μm muscle sections were treated with 3% H_2O_2 for 20 min, then incubated with mouse anti-active caspase-3 antibody (SunLong Biotech Co., Cat. No. SLM33199M) overnight. After washing, they were incubated with Envision detection system (DAKO, Cat. No. K4065) for 30 min, washed by PBS, and incubated with diaminobenzidine (DAB) for 15 min. After washing, they were counterstained with hematoxylin, dehydrated, and cleared in xylene then coverslipped for microscopic examination. Six representative non-overlapping fields were randomly selected per tissue section of each muscle sample for analyzing the mean area percentage of immunoexpression levels of active caspase- 3. Data were obtained using a Full HD microscopic imaging system operated by Leica Application software for tissue section analysis (Leica Microsystems GmbH, Germany).

2.4.5. Determination of total protein content

The protein content was measured according to the method described by Lowry et al. (Lowry et al., 1951).

2.5. Total RNA isolation and quantitative analysis of Mas receptor RT-qPCR

Total RNA was extracted from gastrocnemius homogenate in TRIzol (Invitrogen, USA, Cat. No. 15596026) following the manufacturer's instruction (Promega, Madison, WI, USA). Then, cDNA synthesis was performed as described by Invitrogen, USA, Cat. No. 18080051. Real-time PCR amplification followed by the analysis was performed using Applied Biosystem with software version 3.1 (StepOne™, USA) and data acquisition was made during extension step. Relative expression of studied gene mRNA was calculated using the comparative Ct method. GAPDH was considered as the housekeeping gene to which all values were normalized. Primer sequences (sense and antisense) and GenBank Accession Numbers for Mas receptor and GAPDH are shown in Table 1.

2.6. Detection of phospho-p38 MAPK protein by western blot

Proteins were extracted from gastrocnemius muscle homogenates using ice-cold radioimmunoprecipitation assay (RIPA) buffer supplemented with phosphatase and protease inhibitors. Following centrifugation, proteins were separated by SDS/polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Pierce, Rockford, IL, USA). After transfer, the membranes were washed with PBS then blocked in blocking buffer, followed by incubation overnight at pH 7.6 at 4 °C with antibodies (1:1000, dilution) for p38 MAPK (Cat. No. PA5-17713), phospho-p38 MAPK (Cat. No. 44-684G) and β-actin (Cat. No. PA1-46296) (Thermo Scientific, Rockford, Illinois, USA). After washing, membranes were incubated at 37 °C for 1 h with peroxidase-labeled secondary antibodies (1:4000, dilution). Band intensity was analyzed by ChemiDocTM imaging system with Image LabTM software version 5.1 (Bio-Rad Laboratories Inc., Hercules, CA, USA). Phospho-p38 MAPK protein expression was represented relative to p38 MAPK (as fold change from control).

2.7. Histopathological examination

Gastrocnemius muscle specimens were treated according to Bancroft et al. (Bancroft D, 1996). Scoring of inflammatory cells infiltration was performed according to Vizcaino-Castillo et al. as following: 0 = normal, 1 = scarce cellular infiltrate, 2 = diffuse infiltrate, 3 = abundant infiltrate (Vizcaino-Castillo et al., 2014). Tissue sections were analyzed using a Full HD microscopic imaging system operated by Leica Application software (Leica Microsystems GmbH, Germany).

2.8. Statistical analysis

Data are expressed as mean ± S.D of 6 animals. Statistical comparisons between means were carried out with a one-way analysis of variance (ANOVA), followed by a post-hoc Tukey-Kramer multiple comparison test using GraphPad Prism software (version 6). The statistical significance of difference was considered at $P < 0.05$.

3. Results

3.1. Effect of candesartan, CoQ10 and their combination on ACE2/ angiotensin (1–7)/ Mas receptor axis in rat gastrocnemius muscle

Induction of HLI/R by tourniquet prompted a significant decrease in gastrocnemius muscle ACE2 activity, angiotensin (1–7) level and Mas receptor gene expression by 56%, 53%, and 80%, respectively, as compared to the control group. Treatment with either candesartan (1 mg/kg) or CoQ10 (10 mg/kg) increased significantly ACE2 activity as well as angiotensin (1–7) content and Mas receptor mRNA level compared to the HLI/R group. Interestingly, the combination of candesartan (0.5 mg/kg) and CoQ10 (5 mg/kg) normalized ACE2 activity and angiotensin (1–7) level while it augmented Mas receptor gene expression level by 3.8 folds as compared to the HLI/R group (Fig. 1).

3.2. Effect of candesartan, CoQ10 and their combination on angiotensin II level in rat gastrocnemius muscle

Angiotensin II level was found to be doubled in gastrocnemius muscle after HLI/R when compared to the same muscle of control rats. Treatment of rats after induction of HLI/R with either candesartan (1 mg/kg) or CoQ10 (10 mg/kg) reduced significantly angiotensin II levels by 30% and 39%, respectively. Also, it was found that the combination of lower doses of candesartan (0.5 mg/kg) and CoQ10 (5 mg/kg) normalized angiotensin II level (Fig. 2).

3.3. Effect of candesartan, CoQ10 and their combination on TNF-α and IL-6 levels in rat gastrocnemius muscle

Compared with the control group, the gastrocnemius content of TNF-α and IL-6 increased significantly after induction of HLI/R by approximately 4 folds. Candesartan (1 mg/kg) or CoQ10 (10 mg/kg) reduced significantly the levels of these inflammatory mediators in gastrocnemius of rats with HLI/R. Treatment with the combination of half doses of candesartan and CoQ10 abated TNF-α and IL-6 contents significantly when compared to the HLI/R group (Fig. 3).

3.4. Effect of candesartan, CoQ10 and their combination on MDA content and SOD activity in rat gastrocnemius muscle

HLI/R induced a significant rise in gastrocnemius MDA content by 18.8 folds when compared to the control group. Treatment with candesartan (1 mg/kg), CoQ10 (10 mg/kg) or their combination (0.5 mg/kg of candesartan + 5 mg/kg of CoQ10) normalized MDA content.

On the other hand, there was a significant decline of SOD activity by nearly 78% in gastrocnemius muscles subjected to HLI/R when compared to control muscles. Treatment of injured rats with either candesartan (1 mg/kg) or CoQ10 (10 mg/kg) raised significantly gastrocnemius SOD activity by 3.5 and 3.4 folds, respectively, as compared to the HLI/R group. The combination of lower

Table 1
Genes and PCR primers.

Gene	NCBI gene accession number	Product size	Primers sequence	Reference
Mas receptor	NM_012757.2	2042 bp	F: 5′-CAGATGTCACCGCCCCAAGCA-3′ R: 5′-GTGTTGCCATTGCCCTCCTGA-3′	You et al., 2019
GAPDH	XM_017593963.1	1065 bp	F: 5′-GGTCGGTGTGAACGGATTTGG-3′ R: 5′- ATGTAGGCCATGAGGTCCACC-3′	Fikry et al., 2019

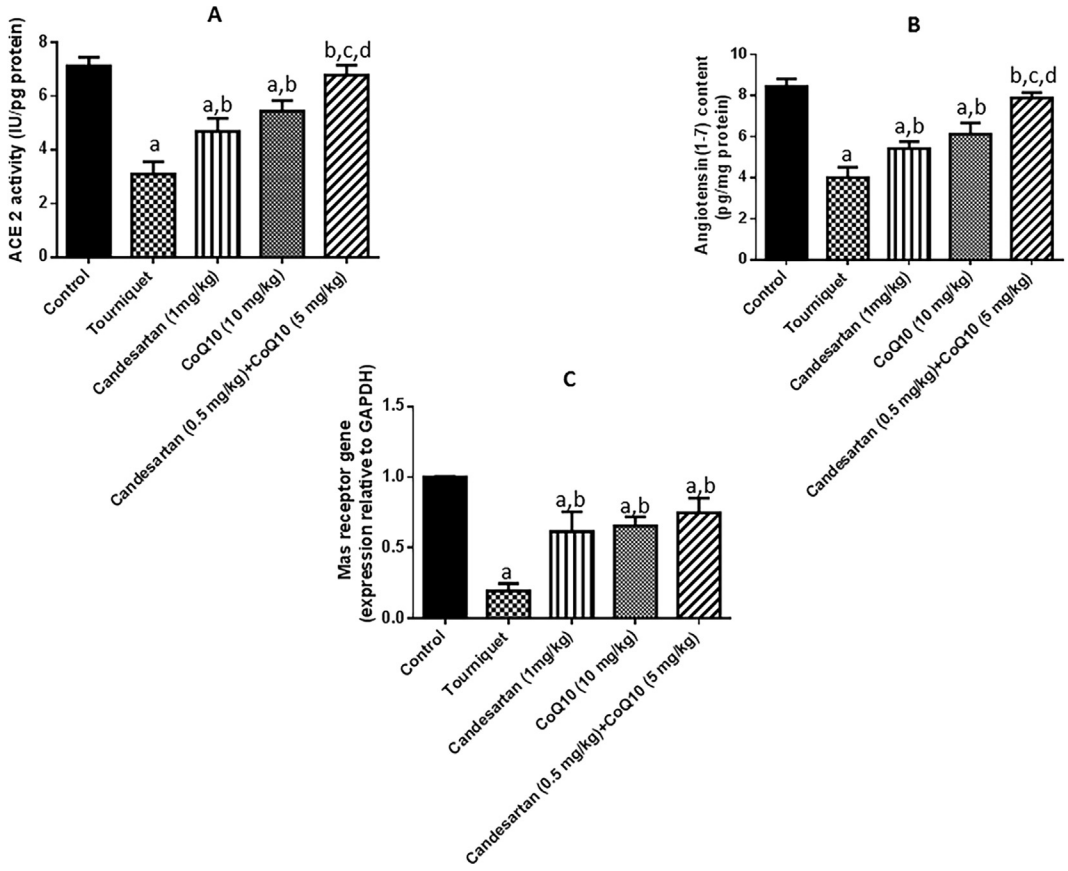

Fig. 1. Effect of candesartan, CoQ10 and their combination on ACE2/angiotensin (1–7)/ Mas receptor axis in rat gastrocnemius muscle. (A) ACE 2 activity, (B) angiotensin (1–7) content, (C) Mas receptor gene expression. Data are expressed as mean ± S.D (n = 6). a, b, c, d: significantly different from control, tourniquet, candesartan, CoQ10 groups, respectively, at $p < 0.05$.

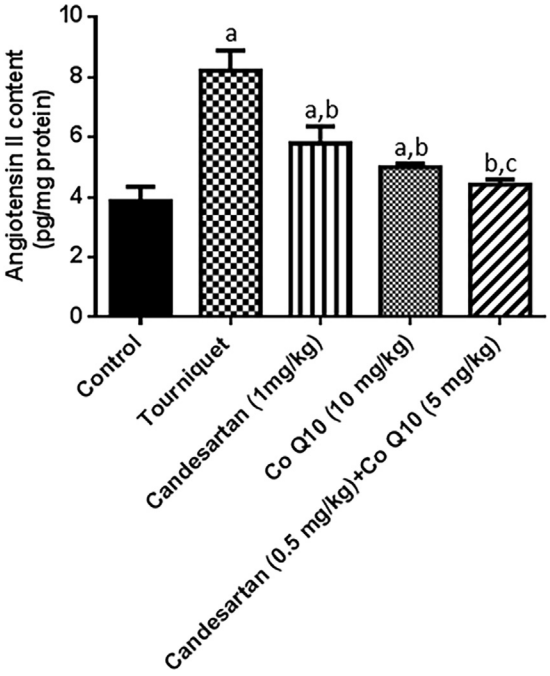

Fig. 2. Effect of candesartan, CoQ10 and their combination on rat gastrocnemius angiotensin II content. Data are expressed as mean ± S.D (n = 6). a, b, c: significantly different from control, tourniquet, candesartan groups, respectively, at $p < 0.05$.

doses of candesartan (0.5 mg/kg) and CoQ10 (5 mg/kg) gave a similar effect to the full dose of each drug alone (Fig. 4).

3.5. Effect of candesartan, CoQ10 and their combination on active caspase-3 expression in rat gastrocnemius muscle

HLI/R provoked a significant increase in area percentage of active caspase-3 immunoexpression in rat gastrocnemius muscle in comparison to the control group. Treatment of rats after induction of HLI/R with either candesartan (1 mg/kg) or CoQ10 (10 mg/kg) reduced active caspase-3 expression by almost 52% and 74%, respectively. The combination of the half doses of both drugs further reduced active caspase-3 expression to 80% (Fig. 5).

3.6. Effect of candesartan, CoQ10 and their combination on phospho-p38 MAPK expression in rat gastrocnemius muscle

Induction of HLI/R caused an increase in the expression level of gastrocnemius phospho-p38 MAPK by 2.5 folds. Treatment of rats with either candesartan (1 mg/kg) or CoQ10 (10 mg/kg) diminished significantly phospho-p38 MAPK protein expression by 28% and 19% when compared to rats subjected to HLI/R. Interestingly, the combination of half doses of both drugs decreased normalized phospho-p38 MAPK when it was administered to rats after exposure to HLI/R (Fig. 6).

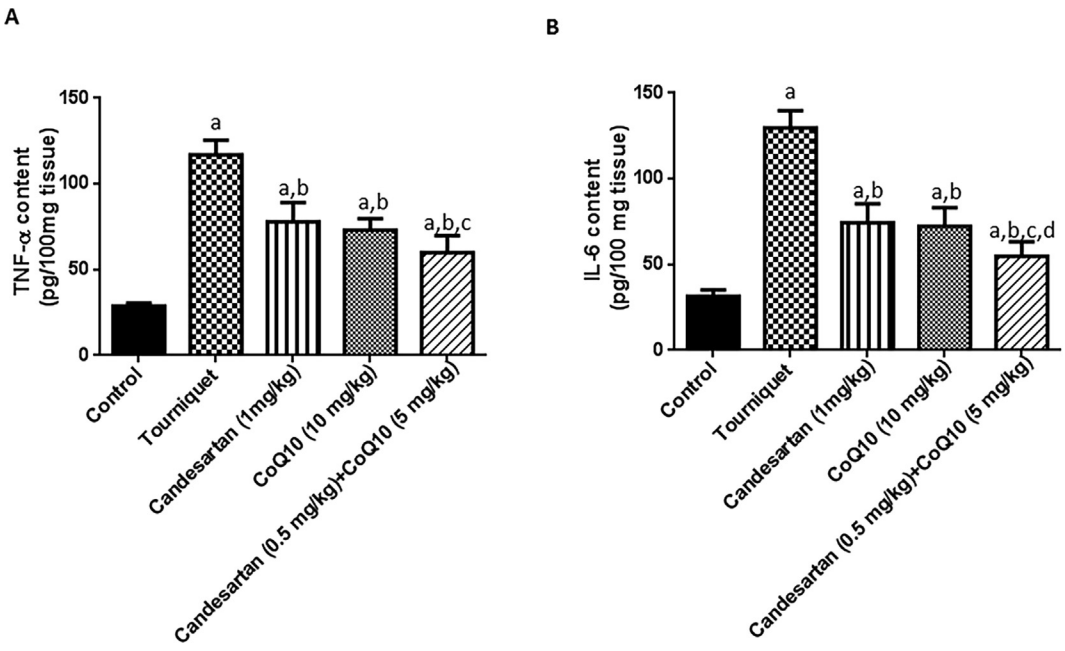

Fig. 3. Effect of candesartan, CoQ10 and their combination on rat gastrocnemius inflammatory markers. (A) TNF-α, (B) IL-6 levels. Data are expressed as mean ± S.D (n = 6). a, b, c, d: significantly different from control, tourniquet, candesartan, CoQ10 groups, respectively, at $p < 0.05$.

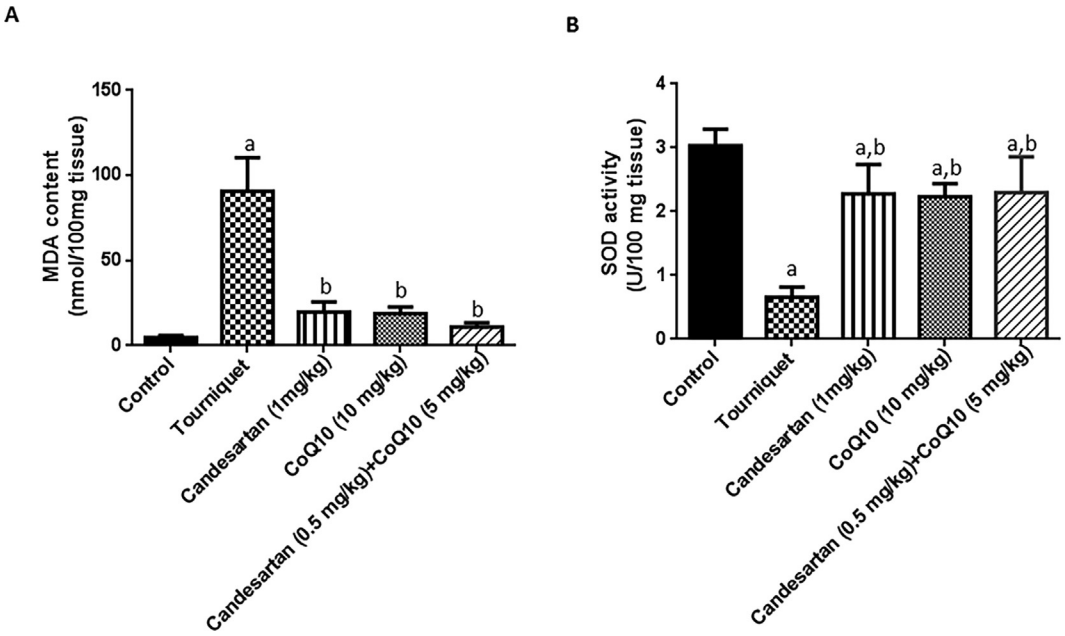

Fig. 4. Effect of candesartan, CoQ10 and their combination on rat gastrocnemius oxidative stress markers. (A) MDA content, (B) SOD activity. Data are expressed as mean ± S.D (n = 6). a, b: significantly different from control, tourniquet, respectively, at $p < 0.05$.

3.7. Effect of candesartan, CoQ10, and their combination on histopathological changes in rat gastrocnemius induced by HLI/R

Massive inflammatory cell infiltration was detected in the sarcolemmal sheath of the gastrocnemius muscle of rats subjected to HLI/R (Fig. 7 (b)). Treatment with either candesartan (1 mg/kg) or CoQ10 (10 mg/kg) diminished significantly inflammatory cells infiltration (Fig. 7 (c) and (d), respectively) while the combination of half doses of both drugs results in a comparable histological aspect with that of control gastrocnemius (Fig. 7 (a)), where no histopathological alteration was recorded (Fig. 7 (e)).

4. Discussion

Efforts to publicize awareness about tourniquet use between civilians have increased in the last decades. Pre-hospital tourniquet application to people exposed to peripheral exsanguination harm was found to reduce the mortality rate in these patients (Goodwin et al., 2019). However, measures to avoid the possible deleterious effects resulting from I/R upon tourniquet application must be taken into consideration. Skeletal muscles, especially glycolytic type, are considered from the organs prone to injury in such situation.

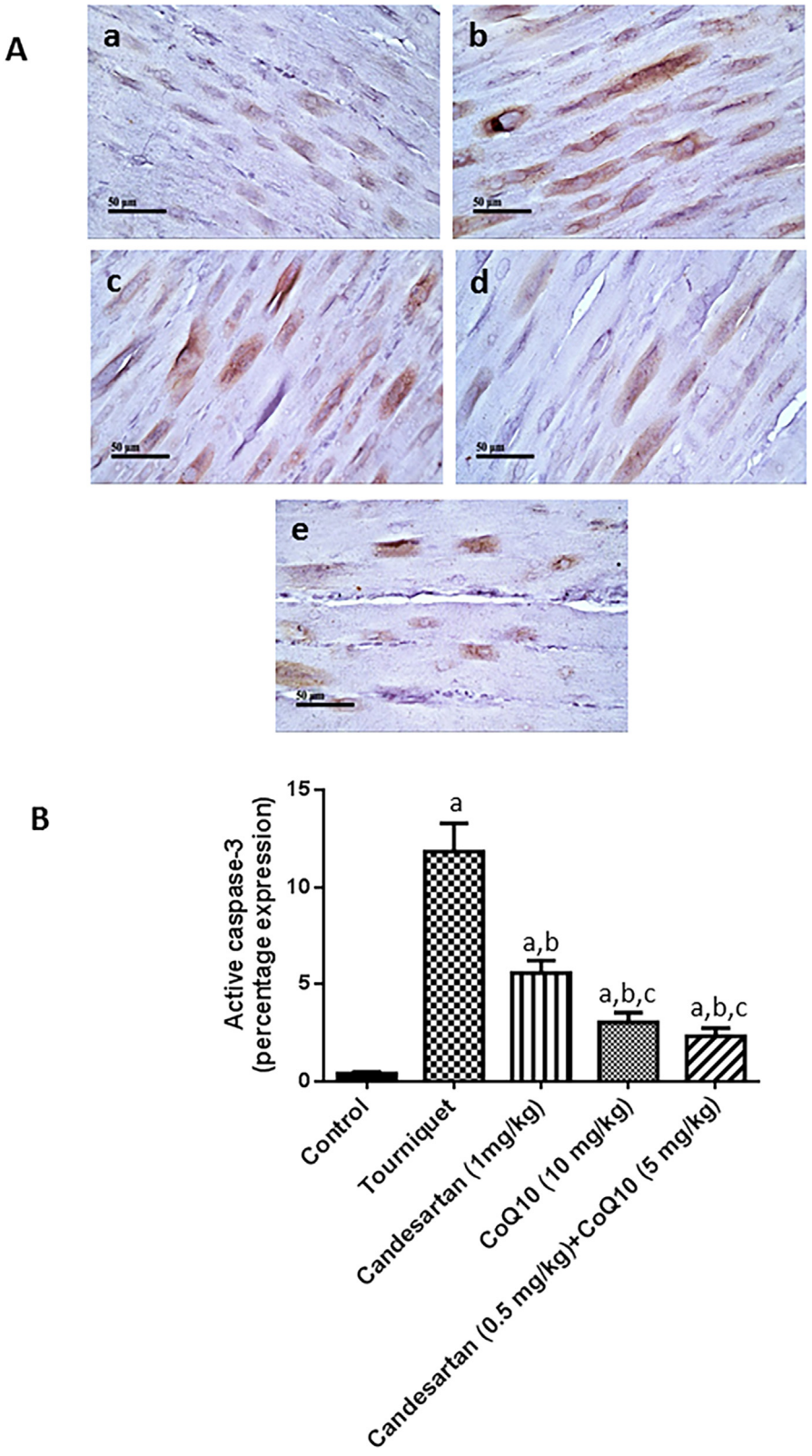

Fig. 5. Effect of candesartan, CoQ10 and their combination on active caspase-3 expression in rat gastrocnemius. (A) Micrographs showing immunohistochemical staining of active caspase-3: (a) control , (b) tourniquet, (c) candesartan, (d) CoQ10, (e) combination groups. (B) Quantification of area percentage of active caspase-3 expression. Data are expressed as mean ± S.D (n = 6). a, b, c: significantly different from control, tourniquet, candesartan, respectively, at $p < 0.05$.

AT1 receptor blockers (ARBs), represented by losartan, proved experimental and clinical efficacy concerning skeletal muscle regeneration (Bedair et al., 2008, Burks et al., 2011, Gharaibeh et al., 2012). Herein, we investigated the effect of the long-acting ARB candesartan, alone and in combination with the promising nutraceutical CoQ10, in a model of rat hind limb tourniquet appli-

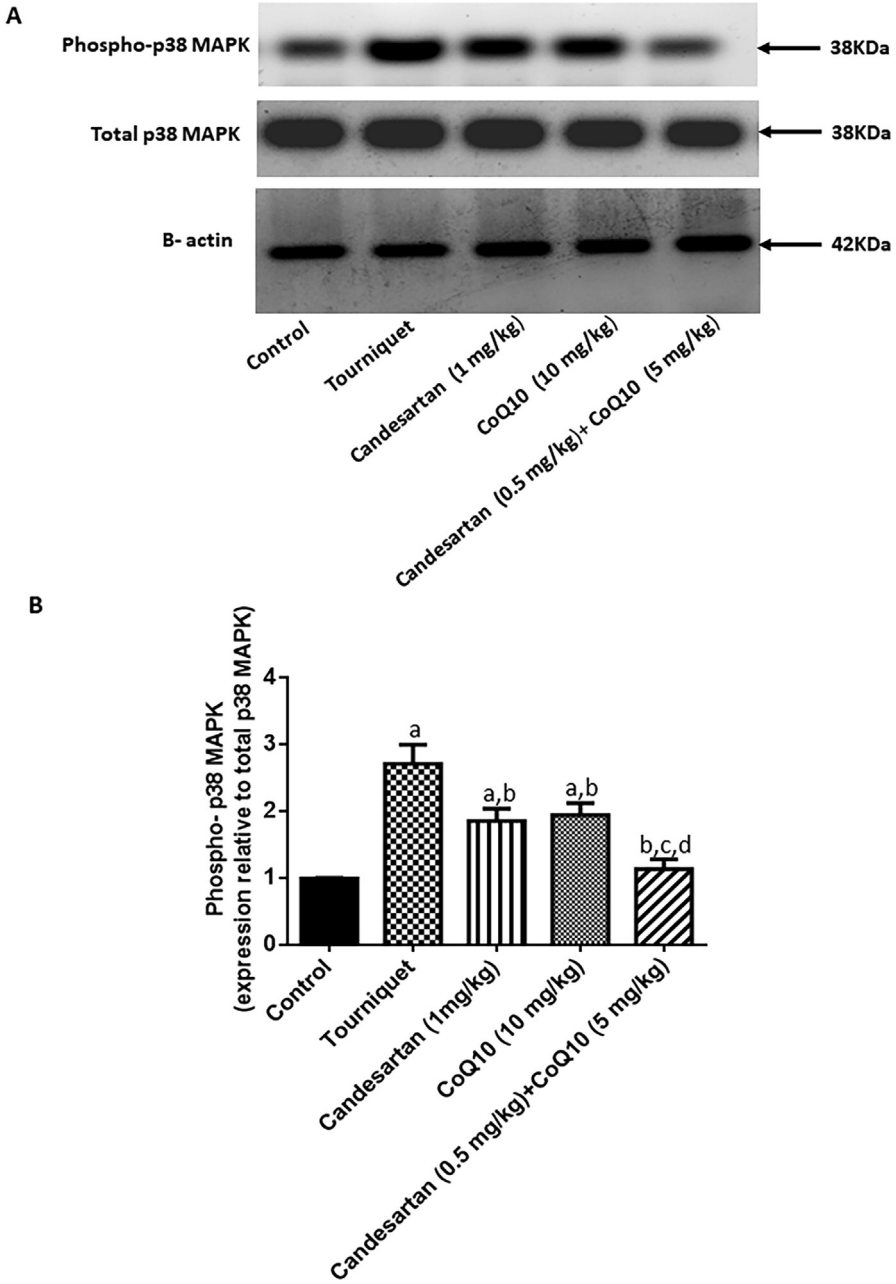

Fig. 6. Effect of candesartan, CoQ10 and their combination on phospho-p38 MAPK expression in rat gastrocnemius. (A) Representative western blots for phospho-p38 MAPK, p38 MAPK, and β-actin proteins expression (B) Quantification of phospho-p38 MAPK protein expression relative to p38 MAPK (presented as fold change from control). Data are expressed as mean ± S.D (n = 6). a, b, c, d: significantly different from control, tourniquet, candesartan, CoQ10 groups, respectively, at $p < 0.05$.

cation. Being an inverse agonist of AT1 receptors, candesartan was shown to inhibit both Ang II-dependent activation of AT1 receptors as well as mechanoactivation (considered as independent of Ang II) (Hong et al., 2016, Zou et al., 2004). Ang II is a well-discriminated mediator produced in tissues damaged by the incidence of I/R. AT1 receptors activation during I/R is well known to trigger NADPH oxidase enzyme complex (NOX2) and subsequent ROS production. This is followed by uncoupling of mitochondrial respiration and further production of ROS by mitochondria. In parallel, inflammation is induced by ROS that initiates NF-κB activation leading to an increase of inflammatory cytokines expression like IL-6 and TNF-α (Powers et al., 2018, Rodriguez-Lara et al., 2018).

Previous studies showed that Ang II administration to mice caused a decline of skeletal muscle mitochondrial content as well as dysfunction followed by muscle atrophy (Kadoguchi et al., 2015, Mitsuishi et al., 2009). Characterization of functional human and mouse mitochondrial angiotensin system by Abadir et al. supports the possible involvement of direct Ang II damaging effect on mitochondria (Abadir et al., 2011).

In the present study, HLI/R induced by tourniquet application caused the elevation of gastrocnemius Ang II, MDA, TNF-α, and IL-6 contents while it decreased SOD activity. Administration of 1 mg/ kg of candesartan ameliorated the oxidative stress and inflammatory deleterious changes affecting the muscle.

CoQ10 plays a crucial role in the mitochondrial respiratory chain in addition to its potent free radicals scavenging properties. CoQ10 deficiency leads to mitochondrial disorders and cellular dysfunction especially in tissues with high energy requirements

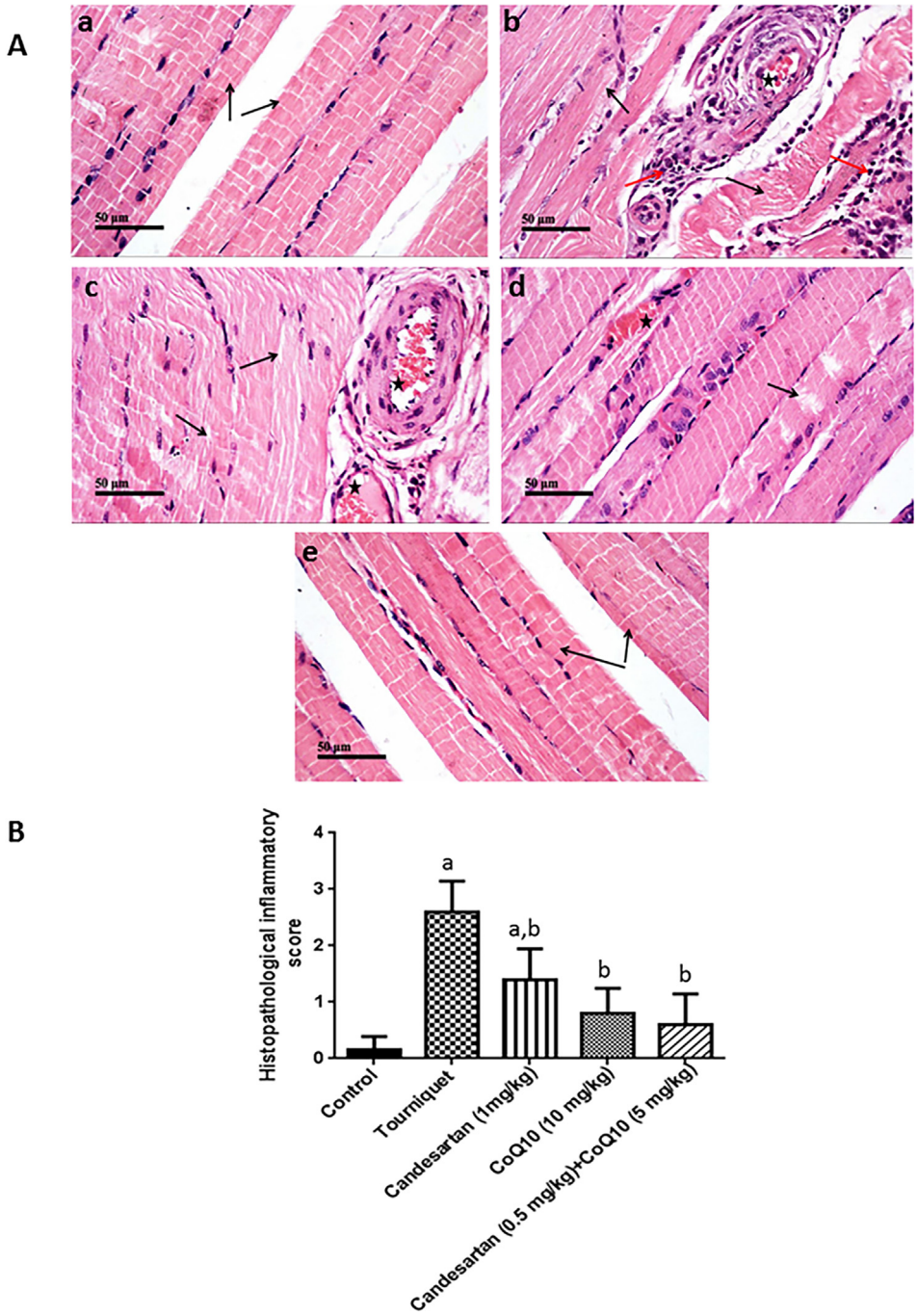

Fig. 7. Representative hematoxylin and eosin staining of rat gastrocnemius showing the effect of candesartan, CoQ10 and their combination on histopathological changes after tourniquet application (scale bar: 50 µm) **(A)**: (a) Normal histological structure and striation of skeletal muscle fibers ***(arrow)*** were recorded in control group with minimal inflammatory cells infiltrates (b) wide areas of degenerated and myofibrillar fragmentation of muscle fibers losing their striations ***(arrow)*** accompanied by intramuscular as well as perivascular mononuclear inflammatory cells infiltrates ***(red arrow)*** with moderate congestion of blood vessels ***(star)*** after tourniquet application (c) significant reduction of inflammatory cells infiltrates records with persistence of congested blood vessels ***(star)*** as well as mild degenerative muscular changes ***(arrow)*** of rat gastrocnemius treated with candesartan (1 mg/kg) (d) mild congestion of intermuscular small blood vessels (bv) ***(star)*** were recorded in rat gastrocnemius treated with CoQ10 (10 mg/kg) with few occasional degenerative changes of muscle fibers ***(arrow)*** and minimal inflammatory cells infiltrates records (e) almost intact morphological features of skeletal muscle fibers ***(arrow)*** of rats receiving combination of candesartan (0.5 mg/kg) and CoQ10 (5 mg/kg). **(B)** Quantification of histopathological inflammatory scores. Data are expressed as mean ± S.D (n = 6). a, b: significantly different from control and tourniquet groups, respectively, at $p < 0.05$.

like skeletal muscles (Romero-Moya et al., 2017, Woodman et al., 2016). Boroujeni et al. demonstrated the protective effect of CoQ10 in a model of HLI/R through suppression of NFκB and TNF-α (Boroujeni et al., 2017). While the action of CoQ10 as an inhibitor of calcium influx and its favorable aspect on cellular damage is well documented (Chang et al., 2012, Okamoto et al., 1995), its effect on classical and non-classical RAS system is not revealed yet.

Herein, our findings show that administration of 10 mg/kg of CoQ10 to rats with HLI/R decreased Ang II gastrocnemius level and improved the subsequent oxidative and inflammatory damages comparably as candesartan.

In addition to the conventional RAS, the presence as well as the anti-inflammatory and antiatrophic role of the non-conventional RAS pathway, ACE2/ Ang(1–7)/ Mas, has been reported in skeletal muscles (Cabello-Verrugio et al., 2015, Riquelme et al., 2014). Kin-

ugawa suggested that the beneficial effects of ARBs in skeletal muscle mitochondrial dysfunction are not only related to AT1R blockade but may involve Ang (1–7) too (Kinugawa, 2017). Candesartan was shown to reduce cardiac damage *via* up-regulation of non-conventional RAS pathway in the myocardium of rats with dilated cardiomyopathy (Arumugam et al., 2012).

In this study, we demonstrate that HLI/R suppressed the skeletal muscle level ACE2 activity, Ang (1–7) level in addition to Mas receptors gene expression. Administration of either 1 mg/kg of candesartan or 10 mg/kg of CoQ10 reversed these changes indicating that the non-classical RAS is involved in the protective action of both drugs. Suppression of ACE2 activity may be a reason for increased Ang II level and in parallel, a decrease of Ang (1–7) level.

It is well recognized that p38 MAPK is activated in response to different stressors including ischemia. Oxidative stress-induced skeletal muscle catabolism was found to be mediated by p38 MAPK phosphorylation (Kim et al., 2009, Meng and Yu, 2010, Rom et al., 2015). Generation of AngII-dependant ROS and phosphorylation of p38 MAPK downstream to NOX activation have been identified in skeletal muscle fibrosis (Cabello-Verrugio et al., 2011, Morales et al., 2012). Besides, it was reported that Ang (1–7) inhibits skeletal muscle wasting through the decrease of p38 MAPK phosphorylation *via* Mas receptor activation (Morales et al., 2015).

Our results are in line with these previous reports. Indeed, HLI/R induced elevation of phospho-p38MAPK. Candesartan (1 mg/kg) and CoQ10 (10 mg/kg) mitigated this elevation probably by diminishing the oxidative stress in gastrocnemius and enhancing ACE2/Ang(1–7)/Mas axis.

Being not far from oxidative stress and inflammation scenario, myonuclear apoptosis is also triggered by Ang II that activates both intrinsic and extrinsic apoptotic pathways resulting in amassing caspase-3 activity (Abrigo et al., 2018, Cabello-Verrugio et al., 2017). On the other hand, Ang (1–7) diminished myonuclear apoptosis and caspase-3 activity in a model of mice skeletal muscle atrophy induced by Ang II (Meneses et al., 2015). Our results are following such findings since HLI/R induced gastrocnemius active caspase-3 expression which was countered by administration of either candesartan or CoQ10. These outcomes appear to be reasonable since both drugs suppressed and enhanced the apoptotic and anti-apoptotic arms of RAS, respectively.

As mentioned above, we didn't only use candesartan (1 mg/kg) or CoQ10 (10 mg/kg) individually in our study, but we combined half doses of these drugs too, aiming to get the beneficial outcome of both drugs while using lower doses. Definitely, the results obtained clarify that 5 mg/kg of CoQ10 boosted candesartan low dose effect on ACE2 activity and Ang (1–7) expression. Similarly, anti-inflammatory and anti-apoptotic actions of candesartan were enhanced.

5. Conclusion

To recapitulate, our study pointed to the benefit of candesartan in a model of tourniquet-induced HLI/R in rats. We demonstrated that in addition to being an ARB, candesartan enhances the non-traditional RAS arm. For the first time, we showed that CoQ10 stimulates also the same pathway in skeletal muscle and magnifies the candesartan effect. However how CoQ10 influences the RAS needs further mechanistic investigations.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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