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(Research Article)

Aliskiren and L-carnitine attenuate isoproterenol-induced cardiac hypertrophy via targeting IL-6/JAK2/STAT3/SOCS3 signaling pathway

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Abstract: This research was designed to assess the inhibitory action of aliskiren, L-carnitine, and their combined treatment on JAK2/STAT3/SOCS3 signaling pathway in cardiac hypertrophy induced by isoproterenol injection in rats. Wistar rats were injected with isoproterenol (5 mg/kg/day) for 15 days for induction of cardiac hypertrophy. Hypertrophied animals were concurrently treated daily with aliskiren (50 mg/kg) and/or L-carnitine (200 mg/kg). Either L-carnitine or aliskiren treatment significantly reduced the elevated relative heart weight with a concomitant reduction in brain natriuretic peptide, creatine kinase-MB, and troponin T in isoproterenol treated animals. Additionally, L-carnitine and/or aliskiren treatment significantly reduced myocardial interleukin-6, lipid peroxidation, and markedly increased glutathione content. Aliskiren and/or L-carnitine treatment also attenuated myocardial fibrosis as evidenced by the significant decrease in myocardial collagen I and transforming growth factor-β1. The biochemical results were further confirmed by the improvement in myocardial histopathological architecture. Interestingly, aliskiren and L-carnitine treatment down-regulated the expression of JAK2, STAT3, and SOCS3 in hypertrophied animals. Conclusively, aliskiren/L-carnitine regimen may ameliorate cardiac hypertrophy induced by isoproterenol through mitigating oxidative stress, inflammation, and IL-6/JAK2/STAT3/SOCS3 pathway.

Keywords: JAK2/STAT3; SOCS3; Aliskiren; L-carnitine; Cardiac Hypertrophy.

1. INTRODUCTION

Cardiac hypertrophy is an intermediate stage prior to heart failure, a highly refractory heart disorder, which represents a driving cause of global mortality [1, 2]. Targeting cardiac hypertrophy was proposed to be a possible strategy to stop the progress of heart failure [3]. However, despite the increased understanding of the pathogenesis of cardiac hypertrophy, evolution in treatment stays stagnant in the last years, mainly due to the participation of many molecular pathways in its pathogenesis. The Janus Kinase/Signal Transducer and Activator of Transcription (JAK/STAT) pathway, is among the eminent signaling pathways implicated in hypertrophy pathogenesis [4]. It is involved in regulation of different genes expression that encodes ligands involved in proliferation, inflammation, immune responses, angiogenesis, and cell death [5-7]. Thus the development of therapeutic approaches that focus on modulation of JAK/STAT pathway is pivotal.

The three master parts of JAK/STAT pathway are; a cell membrane receptor, JAK, and STAT proteins [8]. JAK family consists of four proteins [9], while seven members of STAT are recognized in mammals [10]. JAK/STAT pathway is regulated via the suppressor of cytokine signaling (SOCS) proteins[11]. Different ligands as interleukins, interferon, and growth factors activate JAKs and enhance their kinase activity with its downstream STATs [12]. One of the important ligands that also activate JAK/STAT/SOCS is angiotensin II (Ang II) [13-15], the principal executor molecule of renin-angiotensin aldosterone system (RAAS) and the chief player in the pathogenesis of cardiac hypertrophy [16].

Cardiac remodeling therapy focuses on the inhibition of RAAS to delay the progress of myocardial hypertrophy and consequently heart failure. Aliskiren is a potent renin inhibitor used in management of hypertension and related cardiovascular diseases including myocardial hypertrophy[17-19]. Despite the consensus of the cardioprotective effect of aliskiren against cardiac hypertrophy, its full mechanism has not been fully established yet. Recently, aliskiren was shown to ameliorate isoproterenol-induced cardiac hypertrophy via modulation of calcium calmodulin-

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dependent protein kinase delta isoform expression and apoptosis inhibition[19]. However, other molecular targets for aliskiren remains incompletely explored.

L-carnitine is a natural compound present in most mammalian tissues [20]. L-carnitine primarily is responsible for translocating long-chain fatty acyl-coenzyme A to mitochondria to be degraded by β-oxidation [21]. Indeed, L-carnitine was suggested for treating different cardiac disorders including cardiac hypertrophy, presumably via reducing oxidative stress and inflammation[22-25]. Recently, L-carnitine was documented to modulate STAT3 expression [26].

Up till now, information is unavailable about aliskiren or L-carnitine effect on JAK2/STAT3/SOC3 signaling in hypertrophied heart. Hence, this research was planned to assess the effect of L-carnitine in potentiating the cardioprotective action of aliskiren against myocardial hypertrophy, and to shed the light on their role in targeting JAK2/STAT3/SOCS3 signaling pathway.

2. MATERIALS AND METHODS

2.1. Chemicals

Aliskiren was provided by Novartis Pharma AG (Basel, Switzerland). L-carnitine was obtained from Mepaco-MEDIFOOD (Cairo, Egypt). Isoproterenol was purchased from Sigma-Aldrich Chemical Co (St. Louis, MO, USA). Other used chemicals were of analytical grade.

2.2. Animals

Fifty male Wistar rats (200 ± 20 g) were provided from the National Institute for Research, Cairo, Egypt. Rats were reserved at precise controlled housing conditions. Animals were left for seven days before the start of any experimental processes for acclimatization. Standard rat chow and water were provided to the animals *ad libitum*. The Animal Ethics Committee in the Faculty of Pharmacy, Al-Azhar University, Egypt, approved the experimental design of this research (Approval number: 77/2016).

2.3. Induction of cardiac hypertrophy and treatment protocol

Cardiac hypertrophy was established depending on the method of Chowdhury et al. (2013) [27]. Concisely, animals were daily administrated intraperitoneal (I.P.) injection of 5 mg/kg isoproterenol for 15 days. Control rats were injected I.P. with normal saline for 2 weeks. Rats were haphazardly assigned into 5 groups (10 rats/group) as follow; Group 1 (Control): rats were injected with normal saline; Group 2 (ISO): rats I.P. injected with isoproterenol; Group 3 (ISO+ALS): rats were orally administrated aliskiren (50 mg/kg/day for 2 week) [28] with concurrent I.P. injection of isoproterenol; Group 4 (ISO+L-Car): rats were orally administrated L-carnitine (200 mg/kg/day for 2 weeks) [29] with concurrent I.P. injection of isoproterenol; Group 5 (ISO+ALS+L-Car): rats were orally administrated aliskiren and L-carnitine (the same dose) with concurrent I.P. injection of isoproterenol

After 24 hours of last dose administration, blood was collected under mild anesthesia by retro-orbital sinus puncture. Blood was centrifuged, and serum was collected and kept at −80 °C till utilized for biochemical assays. Animals were sacrificed, hearts were quickly collected, washed, dried, and their weights were measured. Next, parts of the heart samples were homogenized (10% w/v) in phosphate-buffered saline, the clear homogenate was used for measuring different biochemical parameters. Other heart samples were placed in 10% formalin for histological examination while last parts were snap frozen to be used for western blot analysis.

2.4. Determination of relative heart weight

The weights of rats as well as their heart weight were recorded. The relative heart weight was determined and utilized as an evidence for cardiac hypertrophy.

2.5. Enzyme-linked immunosorbent assay (ELISA)

Markers for myocardial damage including serum creatine kinase-MB (CK-MB), troponin T, and myocardial brain natriuretic peptide (BNP) were assessed using ELISA kits (MyBioSource, San Diego, USA) depending on the instructions of manufacturer. Myocardial interleukin-6 (IL-6) was assessed using ELISA kit as stated by the manufacturer's recommendation (R&D Systems Inc., Minneapolis, MN, USA).

2.6. Oxidative stress measurement

Concentration of malondialdehyde (MDA) was measured by thiobarbituric acid method. It is based on the reaction of malondialdehyde with TBA at 98 °C. TBARs were determined colorimetrically using assay kit for lipid peroxidation (MDA) (abcam, MA, USA) depending on the instructions of manufacturer. Myocardial glutathione (GSH) content was measured using commercially available kit (Biodiagnostic, Giza, Egypt).

2.7. Assessment of cardiac fibrosis

Myocardial transforming growth factor-β1 (TGF-β1) was determined utilizing ELISA kit

according to manufacturer's recommendations (MyBioSource, San Diego, USA). Collagen I in heart tissue was measured using rat collagen type I ELISA kits (Cusabio Technology, USA). The colored product absorbance was recorded using a microplate reader set to 450 nm.

2.8. Immunohistochemical analysis

Tissue preparation and immunohistological analysis were performed as described previously [30], using antibodies against rat collagen I. Briefly, heart sections were deparaffinized in xylene and rehydrated in graded ethanol. The antigenicity of the protein was enhanced with microwaves in citrate buffer (PH 6.0) at 98°C for 15 min and washed with PBS. Sections then were treated with 3% hydrogen peroxide for 10 min. After that sections were washed with PBS and incubated with the primary antibody for anti-collagen I (abcam company, Cambridge) at 4°C for 12 hours, washed, and finally stained with polymer HRP detection system. The sections were reacted in a 3-3-diaminobenzidine solution for 60s to visualize immunolabeling, and finally counterstained with haematoxylin and mounted.

2.9. Western blot analysis

Phosphorylation of myocardial JAK2 and STAT3 proteins as well as SOCS3 expression were assessed using Western blotting analysis. After blotting, blocking in 5% bovine serum albumin in Trisbuffered saline containing 0.05% Tween (TBST) was performed. The membrane was processed with primary antibodies against p-JAK2, p-STAT3, and SOCS3 (1:1,000) (Cell Signalling Technology, Beverly, MA, USA) diluted in 1x TBST-buffer for 12 hours at 4 °C, afterwards, membrane was rinsed and incubated for one hour at room temperature with secondary antibody diluted in 1x TBST-buffer (1:10,000) before signal detection using enhanced chemiluminescence (ECL) system. The phosphorylation residue sites of the antibodies that were used in Western blot analysis are the main two tyrosine residues (Tyr1007 and Tyr1008) in the kinase activation loop of JAK2 [31] and Tyr705 for STAT3 [32-34].

2.10. Histopathological examination

Heart tissue was fixed in 10% buffered formalin, dehydrated in ascending dilutions of ethanol, then embedded in xylene-paraffin. Ventricular sections (3 µm) were cut and stained with haematoxylin and eosin (H&E) reagent [35], then visualized under a microscope at 400× magnification.

2.11. Statistical analysis

Analysis of data was done by SPSS (version 21) statistical software. All results were expressed as mean ± S.D. Multiple comparisons were performed using ANOVA followed by Bonferroni multiple comparisons test as a post ANOVA test. Significant differences between compared groups were established at a *P values* less than 0.05.

3. RESULTS

3.1. Aliskiren, L-carnitine, and their combined treatment attenuated macroscopic alteration in isoproterenol-treated rats

Isoproterenol-treated rats appeared weaker and fatigued at the end of the study, meanwhile, animals that recieved aliskiren/L-carnitine combination regimen showed just as lowest of such symptoms. Aliskiren or L-carnitine did not markedly modify body weight gain in hypertrophied animals in comparison to control group. A substantial increase (52%) in heart weight was shown in isoproterenol hypertrophied animals versus normal rats ($p < 0.05$). Aliskiren, L-carnitine and their combination protocol markedly decreased this increase in heart weight by 19%, 10.5%, and 28%, respectively compared to isoproterenol-hypertrophied rats (Table 1).

Table 1: Effects of aliskiren and/or L-carnitine treatment on isoproterenol-induced changes on body weight and heart weight in hypertrophied rats.

Groups	Body Weight (g) Initial	Final	Heart weight (g)
Control	199±2.28	221.8±10.03	0.75±0.05
ISO	216.3±5.95	236±11.40	1.14±0.05*
ISO+ALS	194±5.02	221.2±12.02	0.92±0.07$^{*\pi}$
ISO+L-Car	211.3±4.76	230.5±12.52	1.02±0.04$^{*\pi}$
ISO+ALS+L-Car	204±5.18	233.8±9.02	0.82±0.05$^{\pi\varphi}$

Results are represented as means ±S.D (n=6).$^{*}P< 0.05$, versus control group, $^{\pi}P< 0.05$ versus isoproterenol group, $^{\varphi}P < 0.05$ versus L-carnitine treated group respectively, using ANOVA followed by Bonferroni as a post-ANOVA test.

3.2. Aliskiren, L-carnitine, and their combined treatment alleviate myocardial hypertrophy and ultrastructural changes in isoproterenol-hypertrophied rats

Histological evaluation of myocytes structure was done utilizing H&E staining to follow the impact of aliskiren and/or L-carnitine on myocardial histological architecture after isoproterenol treatment in rats (Figure 1A). In isoproterenol treated group, histological evaluation of the ventricles demonstrated

focal degenerated hyalinized myocardium with focal infiltration of inflammatory cells and myofibroblasts proliferation. Treatment with L-carnitine reduced inflammatory cells infiltration and myofibroblast proliferation in myocardial tissues, meanwhile, treatment with aliskiren amended the aforementioned histopathological changes to a greater extent. The combination of aliskiren and L-carnitine altered all of the previously mentioned histological features, where the heart tissue of the combination protocol showed apparently normal myocardial muscle bundle with no inflammatory cell infiltration. The expression of fibrotic protein collagen I was also assessed by immunohistochemistry (Figure 1B). Collagen I expression was increased in isoproterenol treated group, with progressive subendocardial and interstitial fibrosis (arrow) and myofibroblast proliferation (star). The immunohistochemical staining of ventricular muscle of rat received isoproterenol and L-carnitine or aliskiren showed moderate myocardial cell degeneration and hyalinization with mild to moderate decrease in collagen I immunoreactivity (arrow), while in between degenerated and hyalinized bundle; there is still myofibroblast proliferation (star). Notably, rat treated with aliskiren and L-carnitine combination protocol showed nearly normal myocardial muscle bundle. Figure1C further confirms the hypertrophic effect of isoproterenol, where HW/BW ratio (index for hypertrophy) was markedly elevated by 44.7% in isoproterenol-treated rats compared to normal animals. Meanwhile, aliskiren or L-carnitine treatment significantly reduced HW/BW in isoproterenol-treated rats by about 14.6%, and 11.3%, respectively compared to that of isoproterenol treated animals. Interestingly, L-carnitine co-treatment with aliskiren restored HW/BW ratio to a normal control value (Figure 1C). Isoproterenol-treatment also significantly increased myocardial BNP level by 409% versus control group ($p < 0.05$). Aliskiren, L-carnitine, and their combination protocol significantly lowered BNP level ($p < 0.05$) by 73.9% , 63.9%, and 75.6%, respectively compared to isoproterenol hypertrophied rats (Figure 1D).

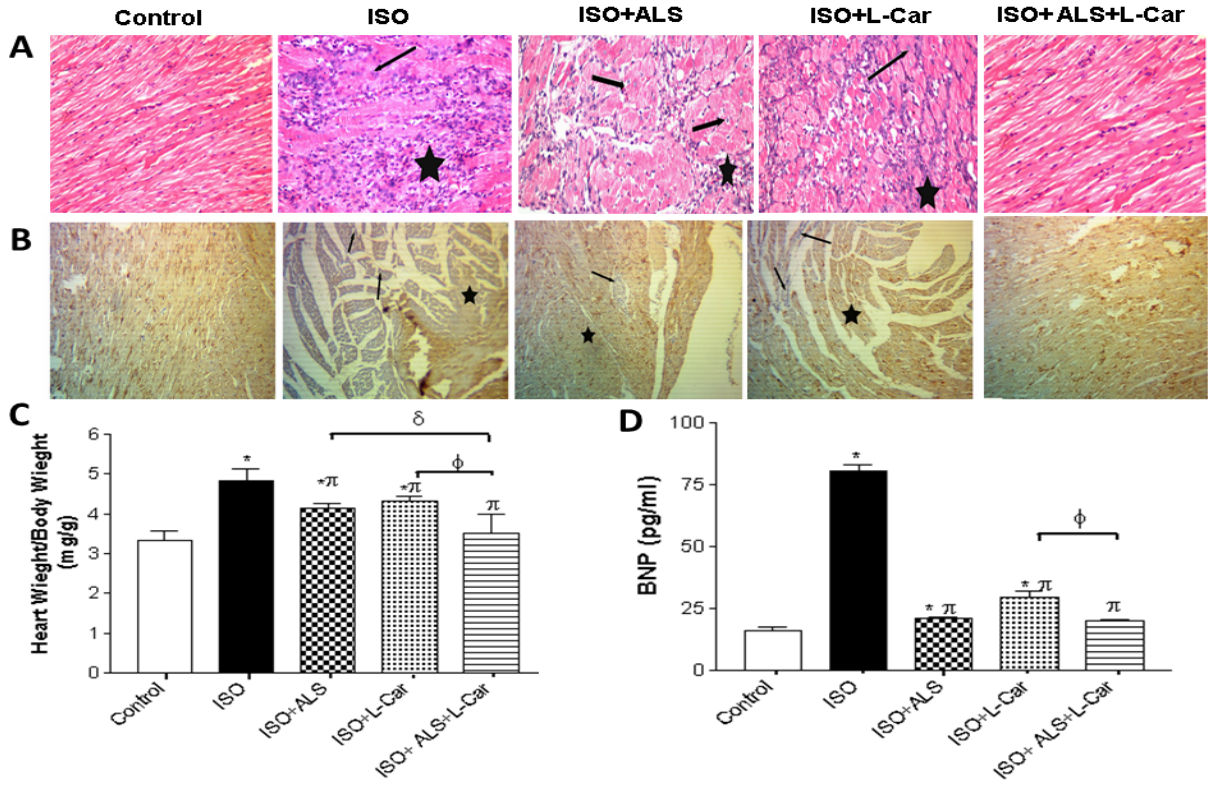

Figure 1: Effect of aliskiren, L-carnitine, and their combination on isoproterenol-induced cardiac hypertrophy in rats. (A) Light photomicrographs of rat ventricular sections stained with H&E. (B) Illustrative images of immunohistochemical staining of collagen in cardiac sections (magnification, ×400; n=3/group), (C) relative heart weight, and (D) myocardial BNP level. Results are represented as mean ±S.D (n=6). *$P < 0.05$ versus control group, $^{\pi}P<0.05$ versus isoproterenol-hypertrophied group, $^{\delta}P < 0.05$ versus aliskiren treated group, $^{\varphi}P < 0.05$ versus L-carnitine treated group, respectively, using ANOVA followed by Bonferroni as a post-ANOVA test

3.3. Aliskiren, L-carnitine, and their combined treatment reduced cardiac injury markers in isoproterenol-hypertrophied rats

In the current study, isoproterenol treatment induced cardiac hypertrophy that was evidenced from the raise in serum CK-MB, and troponin T levels. Figure. 2A and 2B show that, isoproterenol-treatment significantly increased CK-MB (139.5%), and troponin T (960%) compared to normal group. Aliskiren, L-carnitine, and their combination markedly reduced CK-MB by 41.4%, 28.9%, and 46.2% and troponin T levels by 67.5%, 59.3%, and 81.3%, respectively versus isoproterenol-hypertrophied rats.

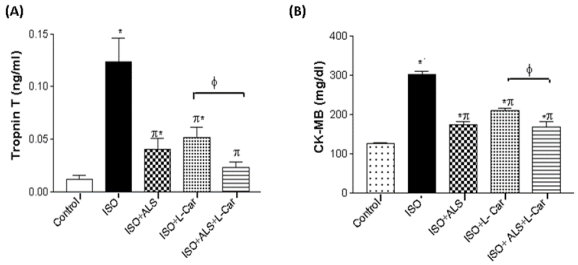

Figure 2: Effect of aliskiren, L-carnitine, and their combination on serum CK-MB (A), and troponin T (B) in isoproterenol-treated rats. Results are represented as mean ± S.D. (n = 6). $^{*}P < 0.05$ versus control group, $^{\pi}P < 0.05$ versus isoproterenol-hypertrophied group, $^{\varphi}P < 0.05$ versus L-carnitine treated group, respectively, using ANOVA followed by Bonferroni as a post-ANOVA test

3.4. Aliskiren, L-carnitine, and their combined treatment reduced oxidative stress and inflammation in isoproterenol-hypertrophied rats

Figure 3A shows that, MDA was markedly greater ($p < 0.05$) in isoproterenol hypertrophied animals (686.2%) in comparison with control group. Aliskiren or L-carnitine treatment markedly reduced lipid peroxidation by 55.9% and 62.9%, respectively versus isoproterenol-treated rats ($p < 0.05$). Additionally, myocardial GSH of hypertrophied rats was significantly reduced by 44.3 % versus normal group. L-carnitine or aliskiren markedly increased myocardial GSH in hypertrophied animals by 38.4% and 50.17%, respectively as compared to isoproterenol-treated animals (Figure. 3B). The combination protocol produced more significant inhibitory effect on oxidative stress, where co-treatment of hypertrophied rats with L-carnitine and aliskiren markedly reduced MDA by 75.2% that was accompanied by significant elevation in myocardial GSH content by 67.5% compared to isoproterenol-treated group.

Additionally, inflammation in the heart tissue of hypertrophied animals was significantly increased as evidenced from the marked rise in myocardial IL-6 level (344.7%) in comparison with normal group. However, treatment with L-carnitine, aliskiren and their combination significantly reduced IL-6 level by 38.2%, 48.6% and 54.7%, respectively as compared to hypertrophied rats (Figure. 3C). Thus, aliskiren/L-carnitine combination treatment significantly mitigated oxidative stress and inflammation to a greater range ($p < 0.05$) than either aliskiren- or L-carnitine- single treatment protocol.

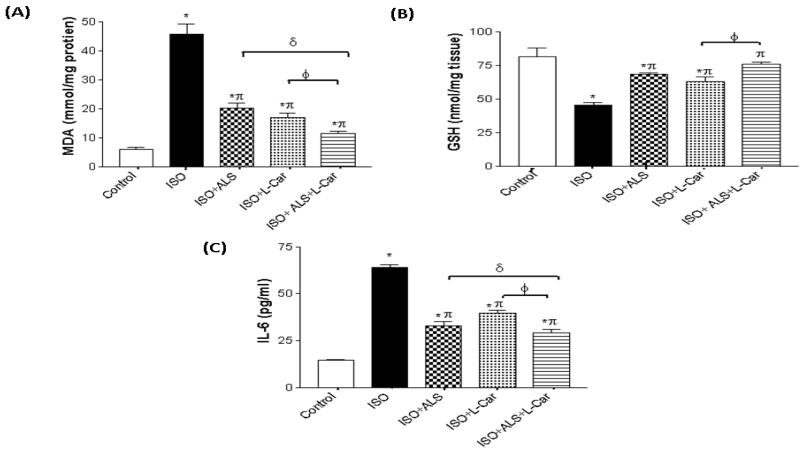

Figure 3: Effect of aliskiren, L-carnitine, and their combination on myocardial MDA (A), GSH (B), and IL-6 (C) in isoproterenol-hypertrophied rats. Results are expressed as mean ± S.D. (n = 6). $^{*}P < 0.05$ versus control, $^{\pi}P < 0.05$ versus isoproterenol-hypertrophied group, $^{\delta}P < 0.05$ versus aliskiren treated group, $^{\varphi}P < 0.05$ versus L-carnitine treated group, respectively, using ANOVA followed by Bonferroni as a post-ANOVA test.

3.5. Aliskiren, L-carnitine, and their combined treatment reduced myocardial fibrosis in isoproterenol-hypertrophied rats

Figures 4 (A) and 4(B) show that, isoproterenol injection significantly increased myocardial TGF-β1 and collagen I content by 224.9% and 217%, respectively in comparison with control rats ($p < 0.05$). Treatment with aliskiren or L-carnitine markedly decreased TGF-β1 levels by 45.9%, and 37.8%, and collagen I by 51.33% and 43.8%, respectively versus isoproterenol-hypertrophied animals

($p < 0.05$). Interestingly, combination regimen significantly reduced TGF-β1 (66.7%) and collagen I (63%) to a greater extent than either L-carnitine or aliskiren single treatment protocol compared to isoproterenol group.

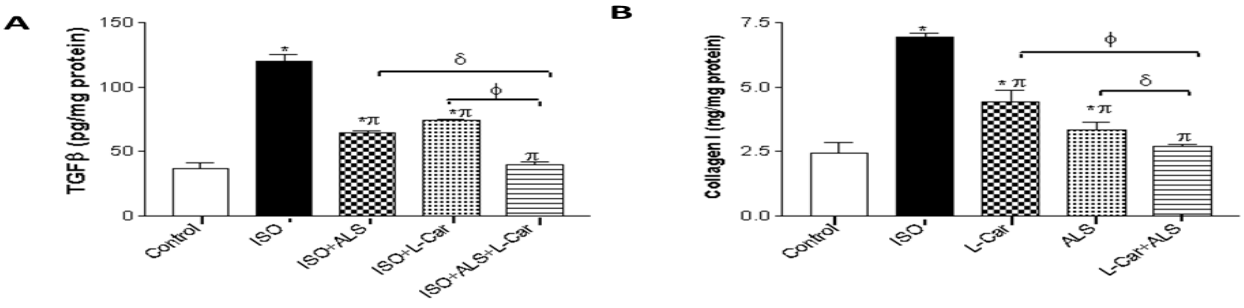

Figure 4: Effect of aliskiren, L-carnitine, and their combination on myocardial TGF-β1 and collagen I content in isoproterenol-treated rats. (n = 6/group). Results are expressed as mean ± S.D. * $p < 0.05$ versus control group, $^{\pi}P < 0.05$ versus isoproterenol-hypertrophied group, $^{\delta}P < 0.05$ versus aliskiren treated group, $^{\varphi}P < 0.05$ versus L-carnitine treated group, respectively, using ANOVA followed by Bonferroni as a post-ANOVA test

3.6. Effect of aliskiren, L-carnitine, and their combined treatment on p-JAK2, p-STAT3, and SOCS3 protein levels in isoproterenol-hypertrophied rats

Figure 5A shows western blotting of p-JAK2, p-STAT3, and SOCS3 protein. Isoproterenol treatment significantly increased p-JAK2 (4.1 fold) and p-STAT3 (5 fold) in myocardial tissue in comparison with normal group. L-carnitine or aliskiren treatment markedly reduced JAK2 phosphorylation by 47.6% and 57.6% and STAT3 phosphorylation by 42.3%

and 50%, respectively versus isoproterenol-treated rats. Interestingly, L-carnitine co-treatment with aliskiren markedly decreased p-JAK2 (70%) and p-STAT3 (71%) in comparison to either single treatment protocol (Fig. 5B,5C). Expression of SOCS3 was also significantly increased after isoproterenol treatment (3.7 fold) compared to control group (Fig. 5D). Treatment with aliskiren or L-carnitine significantly reduced SOCS3 protein expression by 49.2%, and 45.2%, respectively compared to isoproterenol hypertrophied group. Remarkably, the combination regimen significantly lowered SOCS3 expression to a greater extent (60.8%) than either L-carnitine or aliskiren single treatment protocol.

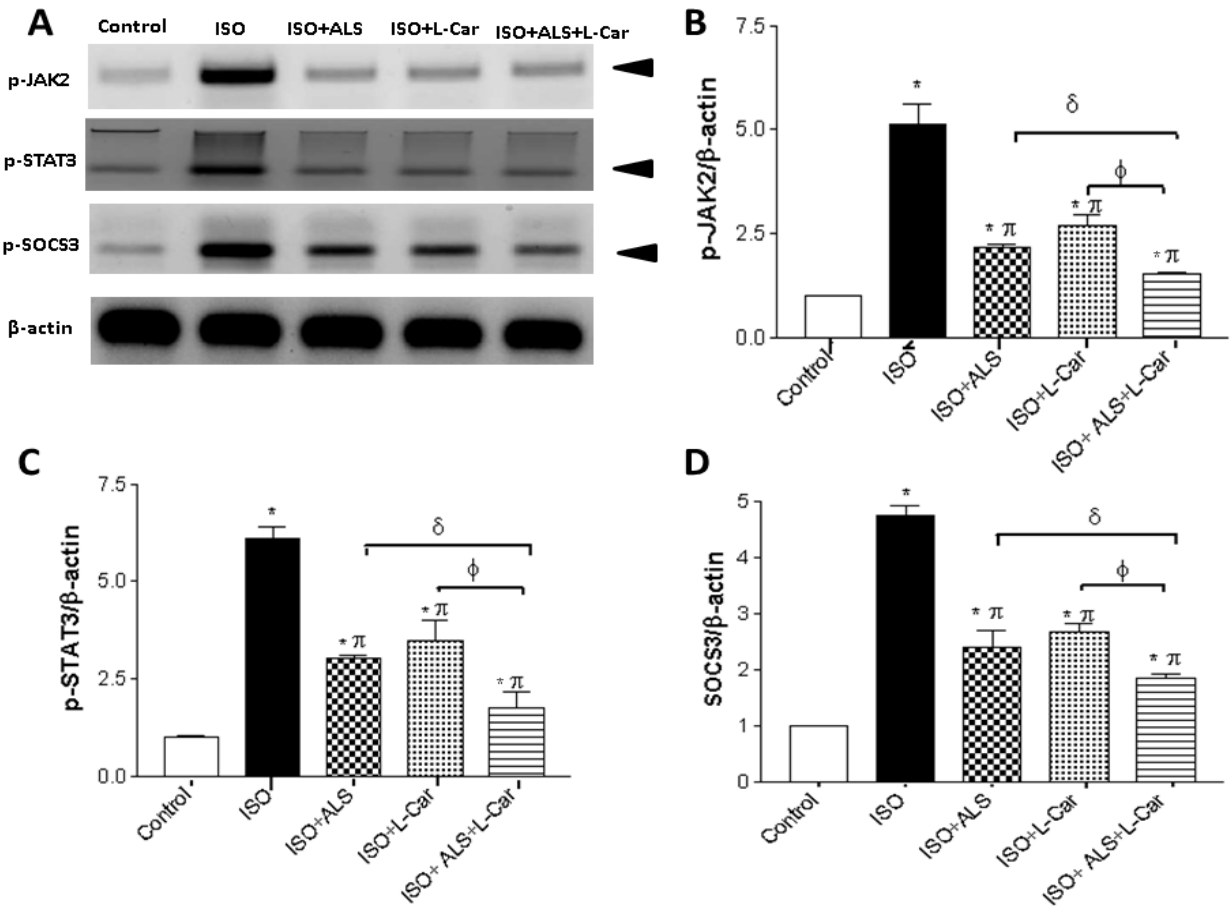

Figure 5: Effect of aliskiren, L-carnitine, and their combination on p-JAK2, p-STAT3, and SOCS3 protein levels in the hearts of isoproterenol-treated rats. A: Western blotting of p-JAK2, p-STAT3, and SOCS3 proteins. B-D: Histogram shows the relative level of p-JAK2, p-STAT3, and SOCS3 proteins. Data are presented as mean ± S.D. (n = 3). * $p < 0.05$ versus control group, $^{\pi}P < 0.05$ versus isoproterenol-hypertrophied group, $^{\delta}P < 0.05$ versus aliskiren treated group, $^{\varphi}P < 0.001$ versus L-carnitine treated group, respectively, using ANOVA followed by Bonferroni as a post-ANOVA test

4. DISCUSSION

For the first time this study revealed that, the ameliorating effect of L-carnitine/aliskiren against isoproterenol-induced cardiac hypertrophy and myocardial fibrosis is partly mediated via inhibition of JAK2/STAT3/SOCS3 pathway. Isoproterenol–treated rats exhibited hypertrophied myocardium that was evidenced from the elevated cardiac index (HW/BW) and increased myocardial injury markers; CK-MB, troponin T, and BNP. Hypertrophic effect of isoproterenol was also associated with myocardial fibrosis indicated by the significant elevation in myocardial collagen I and TGF-β1, and further confirmed by the histological degeneration of myocardial structure. Our data are consistent with earlier reports which showed that, isoproterenol as β-adrenergic stimulant increases the release of cardiac markers indices 4,36,37, cell size and total collagen 38, and fibrosis 39. Aliskiren or L-carnitine single treatment protocol exhibited an ameliorative effect against isoproterenol-induced myocardial damage. Data from earlier studies documented the protective action of aliskiren 19, 40, 41, and L-carnitine 42-45 against experimental and clinical cardiac disorders. The alleviating action of aliskiren on the histopathological alterations was distinct than L-carnitine, and was in accordance with its effect on cardiac markers level. Aliskiren cardioprotective effect depends, at least in part, on its inhibitory action on renin activity and consequently on Ang II level. Meanwhile, L-carnitine protective action is attributed to its antioxidant and anti-inflammatory effects. Interestingly, L-carnitine/aliskiren combination boosts more protective action than single treatment protocols as manifested by the significant lowering in myocardial injury markers, declined HW/BW ratio, and restoration of the normal histopathological structure. The combination regimen also showed more significant anti-fibrotic action than either

aliskiren or L-carnitine single treatment protocol. The increase in collagen I content in myocardial tissue is a characteristic feature of fibrosis that occurs in cardiac hypertrophy and is partially mediated by TGF-β1 [46]. Researchers have documented a rise in collagen I and collagen III synthesis due to β-adrenergic receptor activation of fibroblasts [47]. In heart diseases, cardiomyocytes are lost owing to necrosis, meanwhile, myofibroblasts activation takes place to launch restorative fibrosis as they are the main cells accountable for collagen and extracellular matrix deposition [48]. Myofibroblasts generate Ang II and fibrogenic growth factors that play a critical part in fibrosis and collagen I synthesis [49]. Aliskiren as a renin inhibitor, with its reducing effect on Ang II level, has been demonstrated to decrease deposition of collagen and myocardial fibrosis in different investigational models [50-52]. Mustafa *et al.* [45] showed that, L-carnitine treatment decreased the transformation of fibroblasts to myofibroblasts, and restrained cardiac fibrosis. Also, L-carnitine was shown to reduce Ang II-mediated collagen release [53]. Additionally, the underlying mechanism for the attenuating effect of both drugs on cardiac fibrosis might also be in part due to their inhibitory action on TGF-β1 expression [51-54]. These results are in line with our data in which either aliskiren or L-carnitine treatment markedly reduced myocardial TGF-β1, collagen I expression and deposition, as well as cardiac fibrosis induced by isoproterenol treatment.

One of the key player mechanisms that participate in the progression of myocardial hypertrophy is oxidative stress [55]. Herein, we demonstrated an increase in myocardial MDA content accompanied by a reduction in GSH level in hypertrophied hearts. These data are in accordance with the increase in serum cardiac injury markers that can be elucidated by the elevated oxidative stress and the consequent lipid peroxidation, with a resultant elevation in enzyme outflow from cardiac cells into the serum [56]. Isoproterenol injection and its oxidative metabolism mediate the production of reactive oxygen species (ROS) and depress total cellular antioxidant capability [57]. Additionally, exacerbation of RAAS and the increase in Ang II level is another explanation for the massive production of ROS and the hypertrophic effect of isoproterenol on the heart tissue [58, 59].

Concordantly, treatment with L-carnitine, aliskiren, or their combination suppressed myocardial lipid peroxidation and increased GSH level. These findings are in line with several reports proving the ameliorating action of L-carnitine on oxidative stress and subsequent tissue injury, which might be explained by its free radical scavenging activity either directly or via decreasing its production, preserving mitochondrial electron transport chain efficiency, activating and increasing enzymatic and non-enzymatic antioxidant [22-24,60]. Additionally, L-carnitine is able to protect myocardial integrity via regulating intra-mitochondrial acyl-CoA/CoA percentage resulting in toxic compounds as well as free radicals elimination [61]. On the other hand, aliskiren, as a potent inhibitor of renin, thereby inhibiting RAAS and Ang II production, has been shown to decrease oxidative stress in different experimental models [18,62,63]. In the present study, co-treatment of aliskiren with L-carnitine potentially reduced oxidative stress in untreated hypertrophied rats confirming the suppressive action of aliskiren alone or its combination with L-carnitine on ROS production.

Inflammation represents one of the earliest processes that aid in the development of cardiac hypertrophy [64]. Our data clarified that, aliskiren or L-carnitine treatment significantly lowered IL-6 level in isoproterenol hypertrophied hearts, yet, the combination regimen showed more significant anti-inflammatory action in comparison to either aliskiren or L- carnitine alone. Our data is in line with the evident antioxidant activity seen in the combination protocol, as the main trigger for pro-inflammatory cytokines release are oxidative stress and ROS [65, 66]. Indeed, inflammation and oxidative stress are connected inextricably because they create and magnify each other [65]. Other previous studies revealed that, aliskiren significantly reduced lipid peroxidation, inflammatory markers secretion and expression hence reduce the progression of tissue injury [18,62,67,68]. Meanwhile, L-carnitine has been documented to significantly reduce inflammatory cell infiltration in cardiac tissue [60], as well as serum and myocardial IL-1β and TNF-α levels in coronary artery disease patients [69], diabetic rats [70], and in Nω-nitro-L-arginine methyl ester-treated rats [71]. Therefore, reduced IL-6 level in aliskiren/L-carnitine treated group could be attributed to the enhanced antioxidant and anti-inflammatory action of L-carnitine and aliskiren.

JAK/STAT pathway is a cardio-protective signaling pathway against pathological stresses [72]. However, exaggerated stimulation of JAK/STAT pathway contributes to maladaptive responses and represents a crucial counterpart of the myocardial response to hypertrophy [73, 74]. SOCS3 was known as target gene for the JAK2/STAT3 pathway, forming a negative-feedback loop to stop signal propagation [75]. However, overexpression of SOCS3 intensify myocardial apoptosis and result in immense interstitial fibrosis [76]. On such basis, and to explain

the molecular mechanisms for the protective action of aliskiren and L-carnitine on isoproterenol-induced cardiac hypertrophy, JAK2/STAT3/SOCS3 signaling was assessed as a putative mechanism.

Our findings indicated that, JAK2/STAT3 phosphorylation and the expression of SOCS3 were significantly increased in isoproterenol hypertrophied rats, participating in the pronounced elevation in TGF-β1 and collagen I expression with the subsequent cardiac fibrosis. These data are in accordance with several previous reports [4,7,77]. Actually, several previous studies revealed that SOCS3 expression was increased in many clinical disorders and animal models including cardiac hypertrophy in accordance with the enhanced expression of STAT3 [74,75, 78-80]. The list of inducers of JAK2/STAT3/SOCS3 proteins in the heart is mounting; including several cytokines in addition to Ang II [81-84]. JAK2/STAT3/SOCS3 proteins are crucial participants in Ang II/AT1R signal transduction [5, 84]. Thus, the significant rise in p-JAK2, p-STAT3, and SOCS3 expression could be due to elevated IL-6 level and most importantly to the exacerbated RAAS due to isoproterenol treatment [19,59, 77]. Meanwhile, the anti-inflammatory activity of aliskiren [50,67,68] and L-carnitine [60,69,70], in addition to their established suppressive effect on Ang II level and AT1R expression [50,84-86] assured the positive correlation between the reduced p-JAK2/p-STAT3/SOCS3 expression and the decreased myocardial TGF-β1 level in this study. Thus, our data corroborate to literature, evidencing that, inhibition of JAK2/STAT3/SOC3 pathway might be a crucial mechanism to the mitigating action of aliskiren/L-carnitine therapeutic regimen on myocardial hypertrophy.

5. CONCLUSIONS

Conclusively, our study highlighted that, the cardioprotective effect of aliskiren and L-carnitine might be mediated via their repressive effect on IL-6/JAK2/STAT3/SOCS3 signaling pathway. This effect might be one of the contributing cardioprotective mechanisms besides inhibition of oxidative stress, inflammatory response, and the consequent mitigation of myocardial fibrosis. These findings are supportive for the use of aliskiren and L-carnitine as a protective and therapeutic protocol rescuing the heart from hypertrophy and hence heart failure.

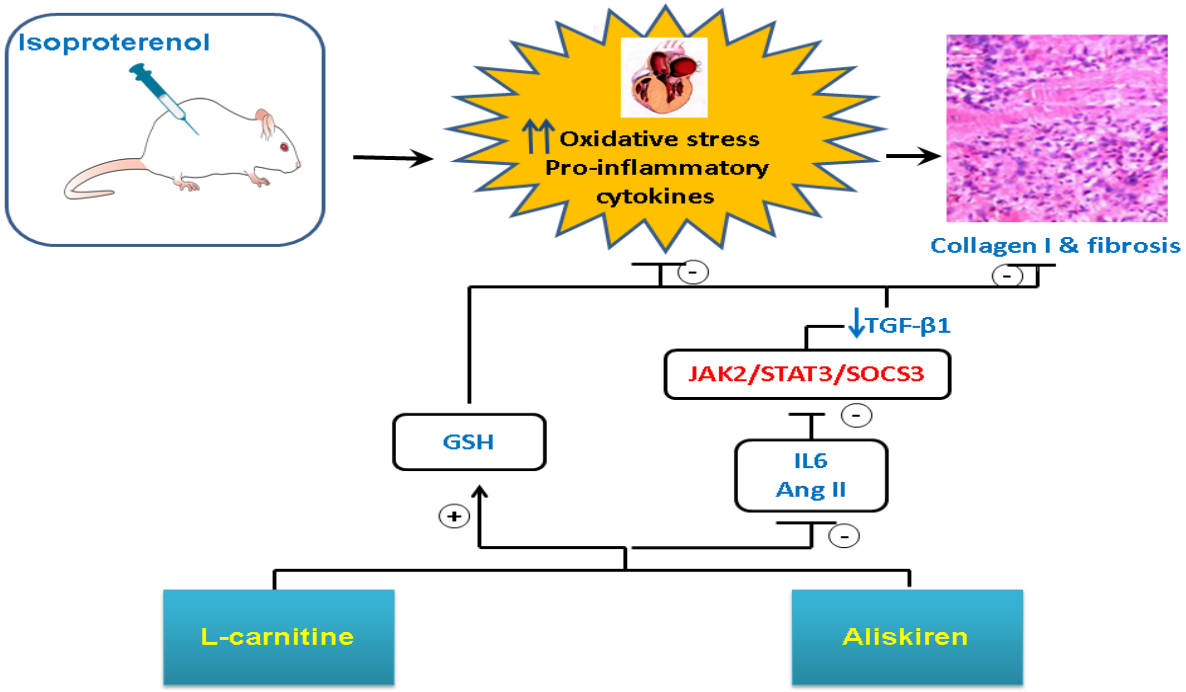

Figure 6: Schematic diagram for the protective effect of aliskiren and L-carnitine against isoproterenol-induced cardiac hypertrophy via targeting IL-6/JAK2/STAT3/SOCS3 signaling pathway.

Conflict of interest: All authors declared no conflict of interest.

Ethical approval: Animal experiments were approved by Animal Ethics Committee of the Faculty of Pharmacy, Al-Azhar University, Egypt. (Approval number: 77/2016).

Author contribution: **MH;** Performed the experiments, collected the data, analyzed the data, performed the graphical and statistical analysis; **NA;** Developed the research idea, designed the experiments, supervised the experiment execution, supervised the data analysis, and wrote and revised the manuscript; **AB;** Supervised the experiment execution, supervised the data analysis and revised the manuscript. All authors read and approved the manuscript, and all data were generated in-house and that no paper mill was used.

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