

Effect of Telmisartan in amelioration inflammatory Responses Induced by Myocardial Ischemia/Reperfusion Injury IN male Mice.

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Abstract

Background: Myocardial ischemia–reperfusion represents a clinically relevant problem associated with thrombolysis, angioplasty and coronary bypass surgery. Angiotensin II may contribute to reperfusion injury by increasing oxidative stress and inflammatory factors . Ang II exerts most of its effects via AT₁Rs. Apoptosis of cardiomyocytes may further be influenced by Ang II.

Objective: This study was undertaken to investigate the potential role of Telmisartan in amelioration of myocardial I/R injury induced by ligation of coronary artery in mouse model.

Materials & method: Adult male Swiss-albino mice were randomized into 4 equal groups. Group (1) sham group: Mice underwent the same anesthetic and surgical procedure as the active control group except ligation of LAD coronary artery. Group(2) active control group: Mice were subjected to regional ischemia for 30 min by ligation of LAD coronary artery and reperfusion for 2 hours .Group(3) control vehicle group (1): Mice in this group injected with DMSO (vehicle for Telmisartan) via IP route & underwent Myocardial ischemia for 30 minutes by ligation of (LAD) coronary artery & reperfusion for 2 hr . Group (4)Telmisartan treated group : Mice pretreated with Telmisartan 0.5mg/kg i.p 30 minutes before ligation of LAD coronary artery.

Results: Compared with the sham group, Levels of TNF- α & IL-1 β , IL-6, caspase 3 and plasma level of cardiac troponin I increased in control group ($p < 0.001$). Levels of Bcl2 decreased in control group ($p < 0.001$). Histologically ,All mice in control group showed a significant ($p < 0.001$) cardiac injury. Telmisartan significantly counteract the increase in myocardium level of TNF- α , IL-1B, IL-6, caspase 3 ,plasma cTnI ($P < 0.001$). Furthermore, the Telmisartan significantly increased in myocardium level of Bcl2. Histological analysis revealed that both Telmisartan markedly reduced ($P < 0.001$) the severity of cardiac injury in the mice underwent LAD ligation procedure .

Conclusion:

The results of the present study reveal that Telmisartan may ameliorate myocardial I/R injury in Male Mice via interfering with inflammatory reactions & apoptosis which induced by I/R injury .

تأثير التلميزارتان في إنقاص الارواء الدموي وإعادته لعضلة القلب وموت الخلايا المبرمج في ذكور الفئران

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الخلاصة

يمثل نقص أرواء عضلة القلب وإعادة الارواء مشكلة ذات صلة سريرية مرتبطة بالجلطات و القسطرة و جراحة تغيير الشرايين التاجية. ألانجيوتنسين الثاني قد يساهم في الإصابة بسبب إعادة الارواء عن طريق زيادة الاكسدة والعوامل الالتهابية. ألانجيوتنسين الثاني يمارس معظم آثاره عبر مستقبلات الانجيوتنسين نوع 1 ، ألانجيوتنسين الثاني يسبب مزيد من موت الخلايا المبرمج في عضلة القلب.

الاهداف :

أجريت هذه الدراسة لمعرفة الدور الوقائي المحتمل للتلميزارتان في تحسين حال الإصابة بالنقص القلبي بالإرواء الدموي/ إعادة الإرواء لعضلة القلب الناجمة عن ربط الشريان التاجي لنموذج الفئران.

الطريقة :

استخدم ثلاثون ذكرا بالغاً من الفئران البيض السويسرية في هذه الدراسة وتم توزيعهم بشكل عشوائي الى اربع مجاميع:
1- مجموعة التظاهر: وهي المجموعة التي مرت بنفس ظروف التخدير وخطوات التجربة لكن لم تجر عليها عملية ربط الشريان التاجي الأمامي النازل الأيسر.

Introduction

Ischemia is a process that occurs when the demand for oxygen exceeds the available supply, most commonly as a result of inadequate blood flow.(1) Restoration of coronary blood flow (reperfusion) by using pharmacological or mechanical interventions following acute myocardial ischemia considered an essential for the salvation of viable myocardium. Paradoxically, reperfusion itself can causes cell damage and cell death mostly by initiating a localized oxidative burst and regional inflammatory response, referred to as “reperfusion injury” (2). Yang *et al.* (1998)⁽³⁾ showed that a brief period of ischemia followed by reperfusion in isolated rat hearts results in an immediate increase in myocardial AT1 receptor expression and myocardial dysfunction, Angiotensin II acts by stimulating pro inflammatory cytokine secretion *In vitro*, angiotensin II induced much greater secretion of IL-6 and TNF- α secretion in co-cultures of cardiomyocytes and fibroblasts than in cultures of fibroblasts

alone, suggesting that paracrine action from cardiomyocytes plays an important role in the production of pro inflammatory cytokines in fibroblasts (4). Zeng *et al.* (2013)⁽⁵⁾ also found that angiotensin-II concentrations in left ventricular tissue increased subsequent to myocardial I/R. Activation of NADPH oxidase, stimulated by angiotensin-II, generates reactive oxygen species, which in turn may act as signal transduction messengers for NF- κ B. Numerous genes, including interleukin (IL)-1, IL-6, IL-8, interferon- γ , TNF-alpha, ICAM-1 and VCAM-1, are stimulated by activation of NF- κ B (6) Telmisartan Is a non-peptide selective antagonist of AT₁R, is often used to treat clinical hypertension and to reduce cardiovascular risk in patients. Telmisartan has shown new pleiotropic actions through induction of PPAR γ activity, providing a potential treatment mechanism for microvascular impedance induced by I/R injury. (7)

Methods and materials

Chemicals and instruments

The materials used in this study are pure Telmisartan powder (Sigma aldrich, USA), ketamine (Hikma, Jordan), Xylazine (RompunTM, 2% vials, Bayer AG, Leverkusen, Germany), ethanol (Fluka, Switzrland) and normal saline (KSA). Mouse tumor necrosis factor- α (TNF- α) enzyme linked immunosorbent assay (ELISA) kits was purchased from Bioscience,. The instruments used in this study were High Intensity Ultrasonic Liquid Processor (Sonics & materials Inc., USA), Vascular Clamp (Biotechno, Germany) and ventilator (Harvard. USA).

Animals

Thirty adult males Swiss Albino mice weighing 28-33g were purchased from Animal Resource Center, the National Center for Drug Control and Researches. The animals were apparently healthy and they were housed in the animal house of College of Medicine/University of Kufa in a temperature-controlled (24 ± 2 °C) room with ambient humidity and alternating 12-h light/12-h dark cycles and were allowed free access to water and standard chow diet until the start of experiments. The mice were left for two weeks without interference for acclimatization. They had no manifestation of any illness upon examination.

Design of the study

Animals were randomly divided into four groups (six Mice/group) assigned as I, II, III, IV, Group I (sham): Mice were subjected for all surgical procedure without ligation of left anterior descending(LAD) coronary artery. Group II (control): Mice were subjected for entire surgical procedure with ligation of (LAD). Group III (control vehicle): Mice were pretreated with 1% DMSO (vehicle forTelmisartan) then subjected to entire surgical procedure with ligation of (LAD). Group IV (Telmisartan treated group): Mice were treated with

0.5mg/kg) (8) via IP injection (9)at 30 minutes before LAD ligation(10) then subjected to entire surgical procedure and ligation.

Surgical procedure and left anterior descending coronary artery (LAD) ligation

Mouse anesthetizes with 100mg/kg ketamine and 5mg/kg xylazine (11).When the animals became unconscious (within 5-10 min), they were placed in supine position with their limbs fixed with stickers to ensure their immobilization during surgery and head extended with traction suture attached to the upper incisor teeth. Hair in the neck and chest regions was shaved and the skin was sterilized. All operative procedures were carried out in clean conditions. Longitudinal nick incision was made; trachea was reached by removing salivary glands via simultaneously pulling each part sideward with forceps. With the same maneuver, the paratracheal muscles on the midline fascia were split to expose the trachea in the larynx area with stay sutures applied to each side of split strap muscle. The trachea was intubated with a cannula sized either 22 G or 20 G according to the weight of animal with the small catheter reserved for the smaller animal. Mechanical ventilation was then achieved by connecting the endotracheal tube to scientific ventilator supplied with 100% oxygen at a respiratory rate of 50/min with a tidal volume of 20 mL/kg body weight [12]. Once steady breathing is established, animal's left limbs were fixed with right side limb, left thoracotomy was made between the 3rd and 4th rib and pericardiotomy was performed by using hemostats or round end scissors to open the space, without cutting the tissue so that the risk of bleeding can be reduced. A chest retractor was positioned within the fourth intercostal space in order to spread the ribs so that the left ventricle (LV) is exposed. Maximal care was taken not to

damage the lung. A wet piece of small gauze, soaked with normal saline was inserted into the thorax to push back the lungs away and expose the heart.

To have a clear view, the pericardium was removed using electronic microscope and LAD was easily detected. The LAD was transiently ligated using a 6-0 prolene suture for a 30-minute ischemic period without exteriorization of the heart (13). The rate of ventilator gradually decreases. When spontaneous breathing was sufficient, decision was made for gentle and careful extubation after freeing the rat from tapes. Finally, the rat was transferred into clean cage oxygenated with 100% oxygen and placed near a heating lamp. Cardiac reperfusion was allowed following 120 minutes of the LAD ligation (13). Microsurgical scissors are used to cut the knot in the ligature. Proper ligation of the LAD was confirmed by observing blanching of myocardial tissue distal to the suture and dysfunction of the anterior wall as observed during the transient LAD ligation. Reperfusion was verified by the return of red color to the myocardial tissue and the demonstration of some recovery of anterior wall motion observed immediately following the transient LAD ligation.

Samples collection

At the end of reperfusion, blood was collected from the ventricles at the apical side. Hearts were cut from their main arteries (aorta and pulmonary artery), rinsed with normal saline to remove any blood, and stored in deep freeze (-20°C to -80°C).

The ventricles were cut from the atrioventricular junction and divided into two parts, lower (apical) and upper parts. The apical parts of the heart were further divided into two parts, one part used for apoptosis study while the other part was

fixed in 10% formalin and processed by routine histological methods and embedded in paraffin blocks (14). For subsequent histological examination, 5 μm -thick horizontal sections were cut and stained with haematoxylin-eosin (H&E).

Samples preparation

Preparation of Sample

The upper parts of the ventricles were washed with cold normal saline to remove any blood, stored in deep freeze (-20°C), and then homogenized with high intensity liquid processor in 1:10 (w/v) phosphate buffered saline that contains 1% triton X-100 and protease inhibitor cocktail (15). The homogenate was centrifuged with 2,500 g at 4°C for 20 min. The supernatant was collected and used in TNF- α determination.

Statistical analysis

Statistical analyses were performed using SPSS 20.0 for windows 7 (IBM, USA). Data were expressed as mean $\pm$ SEM unless otherwise stated. One way Analysis of Variance (ANOVA) was used for multiple comparisons among all groups. Pearson correlation coefficient was used to assess the associations between two variables of study parameters. In all tests; $P < 0.001$ was considered statistically significant.

Result

Telmisartan reduced myocardial inflammatory response

The levels of myocardial cytokines (TNF- α) were found to be significantly elevated ($P < 0.001$) in control group (II) and control vehicle (III) compared with sham group (I). At the same time, cardiac cytokines were significantly decreased ($P < 0.001$) in Telmisartan treated group (IV) with respect to both control and control vehicle groups, (Figure 1)

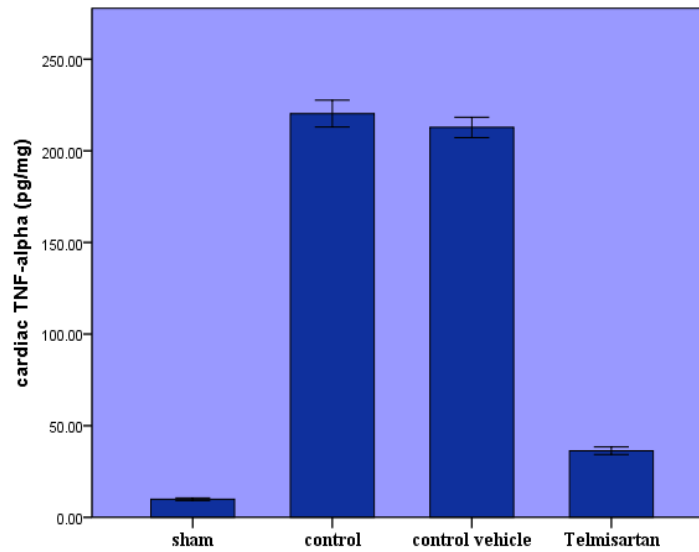


Figure 1. The levels of cardiac cytokines (TNF- α) were found to be significantly elevated ($P < 0.001$) in the control group (II) and control vehicle (III) compared with the sham group (I). At the same time, cardiac cytokines were significantly decreased ($P < 0.001$) in Telmisartan treated group (IV) with respect to both control and control vehicle groups.

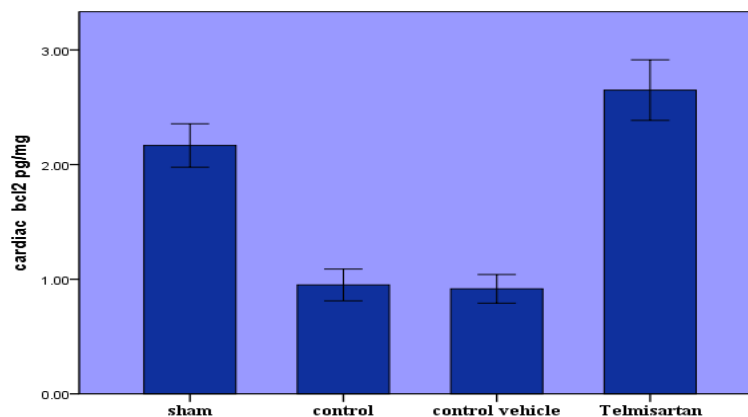


Figure 2. The mean of myocardial BCL2 level (pg/mg of protein) in the four experimental groups at the end of the experiment

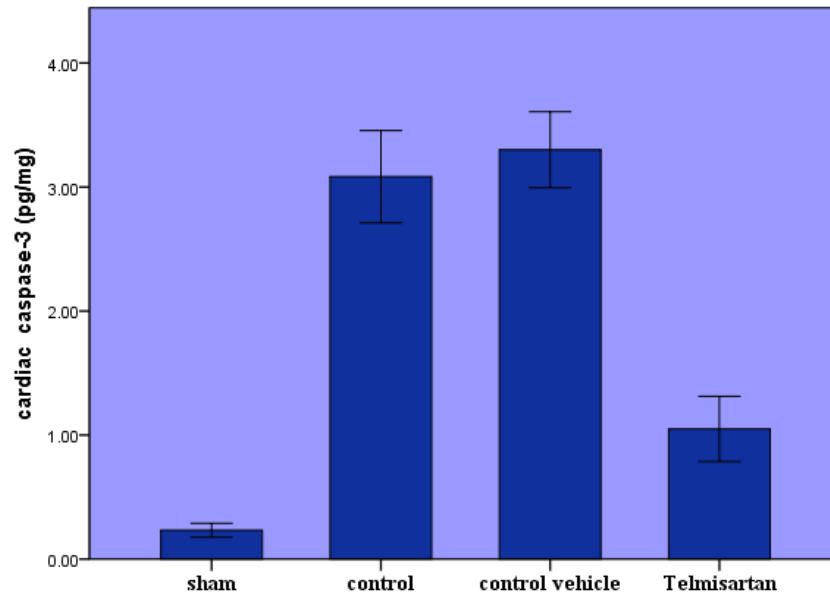


Figure 3 The mean of myocardial caspase-3(pg/mg of protein) in the four experimental groups at the end of the experiment

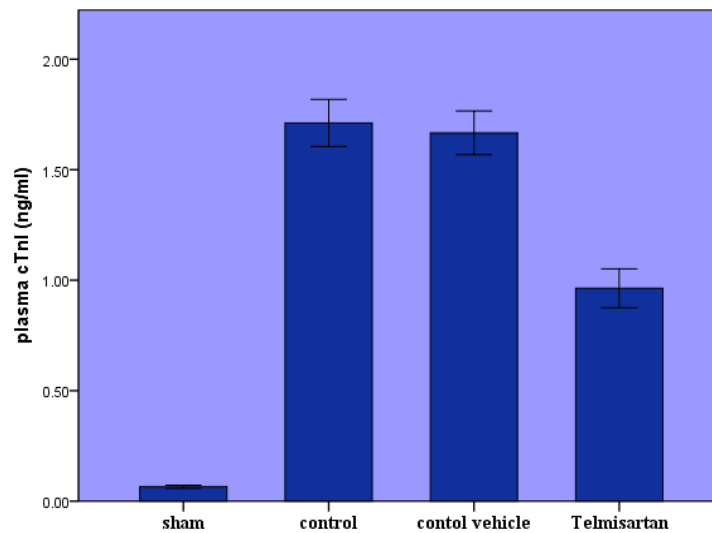


Figure 4 The mean of plasma cTnI level (ng/ml) in the four experimental groups at the end of the experiment

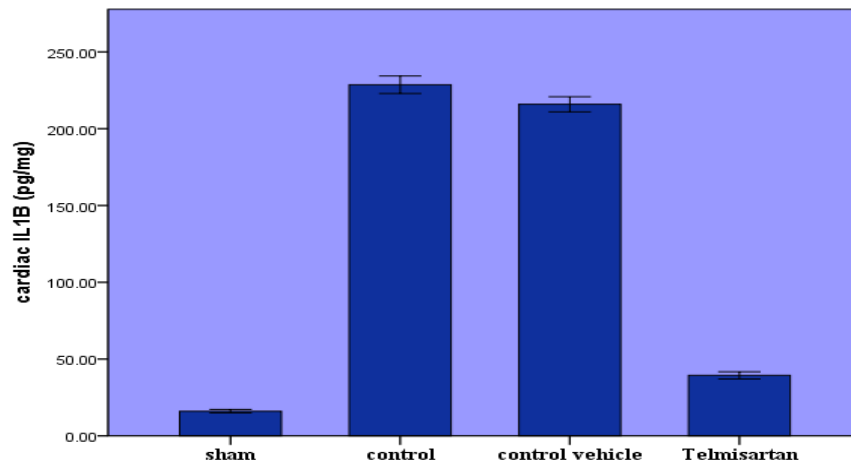


Figure 5 The mean of plasma cardiac IL1B(pg/ml) in the four experimental groups at the end of the experiment.

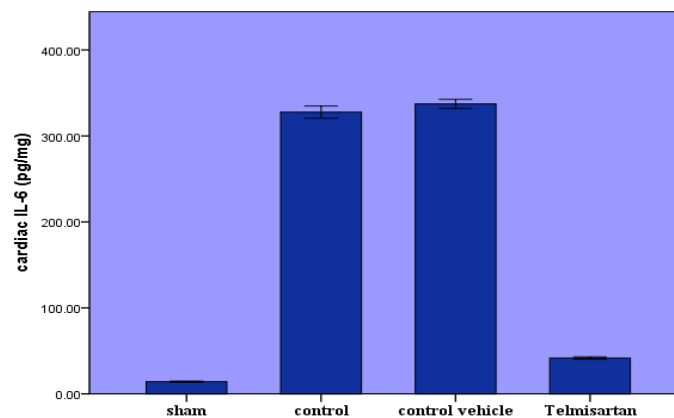


Figure6 The mean of plasma cardiac IL-6 (pg/ml) in the four experimental groups at the end of the experiment

Histopathological findings

A cross- section of heart tissue obtained from the sham group showed normal cardiac structure (score 0); no interstitial edema, no diffuse myocardial cell swelling and necrosis, no neutrophils infiltration, no hemorrhage, no capillary compression and no evidence of apoptosis. There was statistically significant difference between control group and sham group ($P < 0.001$) and the total severity scores of control group showed (66.7%) of the group had sever cardiac injury (score3), (33.3%) had highly sever cardiac injury (score 4). There was statistically insignificant difference between control group (II) and control vehicle (III) and the total severity scores of control vehicle group (III) showed (16.7 %) of the group had moderate cardiac injury (66.7%) had sever cardiac injury and (16.7 %) had highly sever cardiac injury. Treatment of Mice with Telmisartan improved cardiac injury significantly ($P < 0.001$) as compared with control vehicle (III) group and the total severity scores of this group was such that (16.7%) had no damage (score0) and (50%) had mild cardiac injury (score 1) and (33.3%) had moderate cardiac injury (score 2).

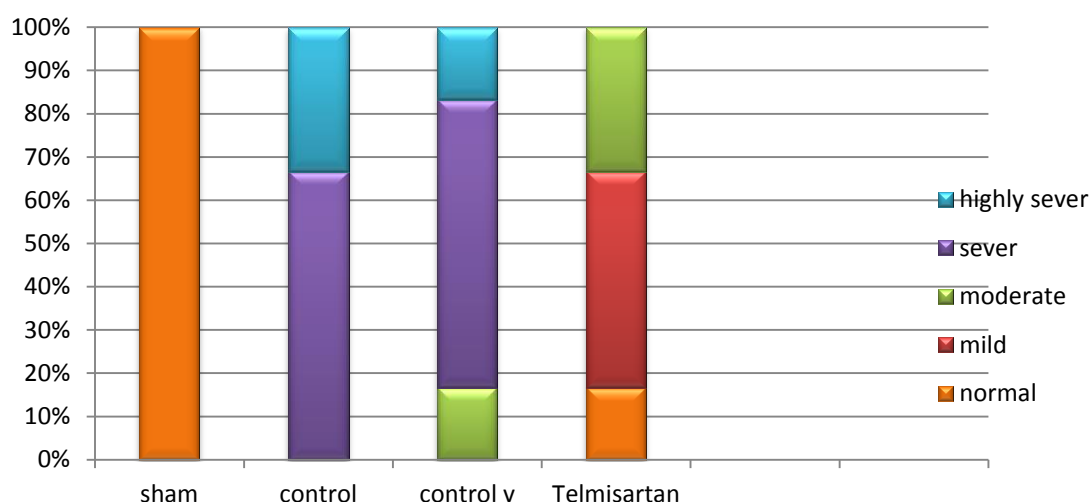


Figure7 Component bar chart the relative frequency of different histopathology grading of abnormal heart changes among the four experimental groups. Score 0 (normal), no damage; score 1 (mild), interstitial edema and focal necrosis; score 2 (moderate), diffuse myocardial cell swelling and necrosis; score 3 (severe), necrosis with the presence of contraction bands and neutrophil infiltrate; and score 4 (highly severe), widespread necrosis with the presence of contraction bands, neutrophil infiltrate, and hemorrhage

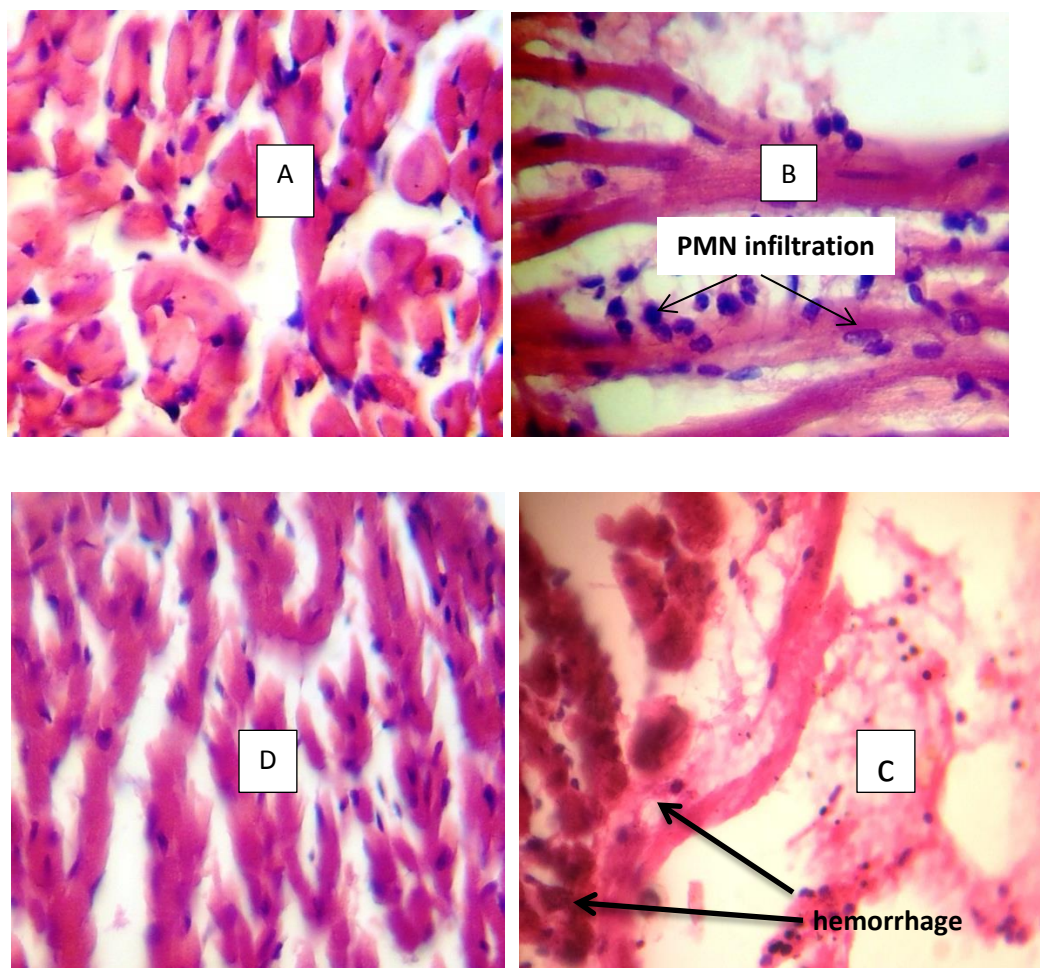


Figure 3. A representative photomicrograph of a section of the heart tissue section stained with Haematoxylin and Eosin (X 40). A, the sham group shows normal architecture (score 0); no interstitial edema, no diffuse myocardial cell swelling and necrosis, no neutrophils infiltration, no hemorrhage, no capillary compression and no evidence of apoptosis. B, cardiac section for the control group showed hemorrhage, necrosis and neutrophil infiltration. C, cardiac section for the control vehicle group showed PMN infiltration and hemorrhage D, cardiac section after treatment with Telmisartan show almost normal cardiac structure.

Discussion

As show in the results, pretreatment with Telmisartan (group IV) significantly ($P < 0.001$) reduced the inflammatory cytokine (TNF- α) levels when compared to the control group (II) and control vehicle group (III).

Reperfusion to the infarcted area is associated with intense inflammatory responses. Inflammatory responses after myocardial I/R injury are detrimental for

cell survival and extracellular matrix integrity via enhanced activation of proapoptotic signaling pathways. (16) Myocardial ischemia has been found to be associated with plasma increased Ang II level (17). Furthermore, Baichun et al. (1998)⁽¹⁸⁾ showed that a brief period of ischemia followed by reperfusion in isolated rat hearts results in an immediate increase in myocardial AT1 receptor expression and myocardial dysfunction, Apoptosis of

cardiomyocytes may further be influenced by Ang II, which binds to the AT1 receptor.(19)

Telmisartan is a unique ARB with selective PPAR- γ -modulating activity which affects nitric oxide bioavailability thus leading to its anti-inflammatory, antioxidant and antiproliferative effects on vascular wall cells (20). **Sukumaran et al.(2011b)**(21) they found that increased myocardial mRNA expressions of inflammatory cytokines [IL-6, IL-1 β , TNF- α and IF- γ] were suppressed by telmisartan treatment group in experimental autoimmune myocarditis induced in Lewis rats by immunization with porcine cardiac myosin.

Takagi et al (2013)(22) concluded that telmisartan therapy is likely effective in reducing IL-6 and TNF- α levels. based on a meta-analysis of nine randomized controlled trials. **Zeng et al.(2013)**(23) found that angiotensin-II concentrations in left ventricular tissue increased subsequent to myocardial I/R. Activation of NADPH oxidase, stimulated by angiotensin-II, generates reactive oxygen species, which in turn may act as signal transduction messengers for NF- κ B. Numerous genes, including interleukin (IL)-1, IL-6, IL-8, interferon- γ , TNF-alpha, ICAM-1 and VCAM-1, are stimulated by activation of NF- κ B(24).Several studies have suggested that Ang II involved in apoptosis For example, **Pollman et al. (1996)**(25) showed that Ang II can directly antagonize NO donor- and cGMP analogue-induced apoptosis via activation of AT1R.

Li et al.,(1999)(26) observed that the effect of Ang II were significantly attenuated by a specific AT1R blocker, indicating and that it is the AT1R activation mediates the pro-apoptotic effects of Ang II.

Treatment of mice with Telmisartan ameliorates heart injury significantly ($P < 0.001$) as compared with induced untreated group. The scores of the control group

showed a severe myocardial injury while the score of Telmisartan treated group showed amild injury.

Iqbal et al (2008) (27) studied the possible protective effect of Telmisartan against doxorubicin induced cardiotoxicity and they found that myocardium of rats pretreated with telmisartan appeared nearly normal. **Zeng et al (2013)**(23) Show the muscle fibers of the I/R and GW9662 Groups (PPARG antagonist) had extensive edema, necrosis, and breakage, and a large number of dissolved myocyte nucleoli, Also, the number of PMNs infiltrating the area of the reperfused myocardium was increased in the I/R, In the telmisartan, telmisartan–GW9662, and candesartan groups, the muscle fibers showed less breakage and edema, along with fewer ruptured nucleoli. There was also significantly fewer PMN infiltration in the myocardium of the telmisartan. Furthermore, there were significantly fewer PMNs infiltrating the myocardium in the telmisartan group compared with the telmisartan–GW9662 and candesartan groups. **Chang et al.(2013)**(28) found that the activity of caspase-3 in the myocardium was significantly greater in I/R group rats than in sham-group rats. **Li et al.,(2013)** reported that cardiac caspase-3 protein expression significantly increase after 30 min LAD occlusion with 2 or 3 hours reperfusion **Tanaka et al.2004**(29) **Fan et al.,(2013)**(30) found that Bcl-2 (anti-apoptotic) protein levels were significantly lower in control mice in hearts subjected to 30 min myocardial ischemia and (30 min of reperfusion)

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