Original Article	**Potential Protective Effect of Pentoxifylline on Myocardial Injury Induced by Ischemia Reperfusion in Rat Model: A Histological and Immuno-Histochemical Study**

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ABSTRACT

Background: Although, myocardial reperfusion is a pre-requisite to salvaging viable myocardium, the process of restoring coronary blood flow can paradoxically induce myocardial injury and cardiomyocyte death. A number of new therapeutic strategies are currently under investigation for preventing myocardial reperfusion injury and to improve clinical outcomes.

Aim of the Work: This study was performed to assess the potential benefit of pentoxifylline in the reduction of myocardial injury induced by ischemia/reperfusion.

Material and Methods: In this study, 30 adult male albino rats were divided into 3 groups; control group, group II: subjected to ischemia (temporary occlusion of the left anterior descending coronary artery for 45 minutes) followed by reperfusion (for 120 minutes), and group III: single dose of pentoxifylline (40 mg/kg/bw) was injected intraperitoneal, 15 minutes before induction of ischemia/reperfusion. Samples from left ventricle from all animals were excised immediately, at the end of experimental time, and processed for examination by light and transmission electron microscopes. Also, immunohistochemical examination using caspase 3 and Anti TNF-α antibodies was performed.

Results: Histological assessment revealed that the pretreatment with pentoxiphilline protected the myocardium against ischemic/reperfusion induced injury; as demonstrated by improvement of histological structure of cardiac myocytes, minimal edema, hemorrhage and fibrosis. No cellular inflammatory infiltration. Also, marked reduction of cardiomyocyte necrosis, indicating strong anti-apoptotic effect of pentoxiphilline, and weak expression of TNF-α, indicating strong anti-inflammatory effect of pentoxiphilline.

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Key Words: Cardiac muscle, ischemia reperfusion, pentoxifylline.

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INTRODUCTION

One of the most major life-threatening conditions is the cardiovascular diseases. It represents a main cause of mortality all over the world. Previously, adult heart was classified as one of the post-mitotic organs which have no ability for self-regeneration. So, cardiac cell death has received a great deal of attention over many years[1], and the cardiovascular disease is considered the hall mark of clinicians' especially acute myocardial infarction (MI)[2]. Coronary artery stenosis is the main cause of impaired cardiac blood supply and leads to Ischemic heart diseases, which are manifested as angina pectoris, acute infarction (MI), and chronic ischemia or sudden death[3]. Although a wide variety of protocols have been conducted for the treatment of cardiovascular diseases, but no certain drug was reported to have a great effective capacity. Although Primary Percutaneous Coronary Intervention is best of choices during treatment of patients with acute MI, but the resulted ischemia reperfusion injury is considered to be more harmful to the myocardium[4]. The underling mechanisms are unclear, but recanalization of the coronaries and dissolving the atherotic plaque is considered the main factor for induction of ischemia reperfusion injury[5].

Myocardial ischemia and reperfusion cause coronary vascular injury involving both the large

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epicardial arteries and the microcirculation[6]. Myocardial ischemia releases multiple inflammatory mediators which cause vascular injury[7]. Despite many pharmacotherapies and considerable progress in revascularisation techniques, current therapies are unable to replace dead cardiomyocytes (CMs) and largely irreversible cardiac dysfunction ensues and many patients progress to heart failure after acute myocardial infarction (MI)[8,9].

A number of investigation are currently under trials to limit the harmful effect of reperfusion injury and to improve myocardial function.

Pentoxifylline (Ptx), is a synthetic methylxanthine, which was first used in 1984 for the treatment of chronic claudication resulted from occlusive arterial disease[10]. Multiple pharmacodynamic effects of Ptx were reported to minimize the tissue injury and protect against ischemia. One of these primary mechanisms is increased RBC deformability and decreased blood viscosity[11]. Also, Ptx has anti-inflammatory properties, and inhibits the production of proinflammatory cytokines. Moreover it reduces accumulation of Tumor Necrosis Factor alpha (TNF-α)[12,13]. In multiple studies, it was stated that the hemorheological and anti-inflammatory activities of Ptx were responsible for its therapeutic effects[14]. Ptx can immensely increase recovery in a variety of organs, including the brain, heart, intestines, testes and skeletal muscle, during ischemia reperfusion injury[15].

AIM OF THE WORK

The current study aimed to investigate the effective therapeutic role of Ptx on myocardial ischemia/ reperfusion injury induced in a rat model, to evaluate the histo-pathological picture and demonstrate its anti-inflammatory and antiapoptotic effects.

MATERIAL AND METHODS

Drugs

Pentoxifylline was purchased from Sigma Aldrich (USA)

Experimental Animal Groups

Thirty adult male albino rats weighing 200g-250g were gained from Nile Pharmaceuticals Company (Cairo, Egypt), and were housed in separate cages. Animals were allowed to have rat chow and water ad libidum. All procedures were done according to the National Institute of Health guide and by the agreement of the Ethics Committee of Ain Shams University, Faculty of Medicine. (NIH Publication No. 85-23, revised 1996).

In this study, the animals were divided into 3 groups: 10 rats each.

Group I: (control group) is further subdivided into 2 subgroups: 5 rats each

- Subgroup Ia: left without any intervention.
- Subgroup Ib: used as sham group.

Group II: (I/R animal model) Rats were subjected to induction of Ischemia/reperfusion without administration of any medication.

Group III: (I/R + PTX ttt animals) Rats were injected intraperitoneal by Ptx , 15 minutes before ligation of left anterior descending artery (before induction of Ischemia/reperfusion)

Induction of Ischemia/reperfusion

The rats were anesthetized with urethane at a dose of 1.2g/kg dissolved in water by intraperitoneal injection. Briefly; animals were intubated with a rodent ventilator at 70-80 breaths/min. The hearts were fully exposed in the thoracic cavity by performing median sternotomy and incision of the pericardium. A ligature was placed around the left anterior descending artery (anterior inter-ventricular artery) and was ligated for 45 min followed by reperfusion for 120 min. Occlusion was confirmed by immediate blanching of the infarcted area[16].

Ptx was given as a single dose of 40 mg/kg/bw dissolved in 1mm saline and given once by intraperitoneal injection 15 minutes before ligation of left anterior descending artery[17].

The sham animals were exposed to the same procedure; the thoracic cavity was opened and the heart was exposed, but without any intramyocardial sutures or ligation of left anterior descending artery.

At the end of experiment, heart slices from the affected area were excised and processed for histological analysis.

Tissue processing for light microscopic examination

10% buffered formalin was used for fixation of tissue samples overnight. Dehydration in ascending grades of alcohol followed by

embedding in paraffin blocks was done. 3mm sections were cut and stained with hematoxylin-eosin (H&E) stains[18]. Sections were examined for morphological changes.

Tissue processing for transmission electron microscopic examination: another heart slices were cut into 1mm3 cubes and were fixed immediately in 2.5% glutaraldehyde (pH 7.3) at a temperature 4°C. 1% osmium tetroxide was used as a secondary fixative for 2 hours followed by dehydration in ascending grades of ethyl alcohol. Tissue were embedded in epoxy resin blocks and 1µm Semithin sections were cut and stained with toluidine blue. Examination by an Olympus light microscope was done[19]. Ultrathin sections were stained with uranyl acetate and lead citrate. Sections were examined and photographed using a JEOL JEM 1010 electron microscope (Jeol Ltd; Tokyo, Japan).

Tissue processing for Immunohistochemical stains

For immunohistochemistry, a standard immunoperoxidase technique was used. Endogenous peroxidase activity was quenched with 3% H_2O_2 for 30 min in the dark followed by a wash with phosphate buffered saline. Sections were subjected to antigen retrieval followed by incubation with the primary antibody: rabbit anticleaved caspase-3 monoclonal antibody (mAb) (diluted 1:100, cat. no PAA626Ra02, Uscn Life Science Inc, China)[20], and Anti-TNF (rabbit polyclonal IgG, 100 µg/ml, 1:50 dilution; Santa Cruz Biotechnology, Inc., USA)[21]. The sections were rinsed in PBS and left overnight (1:200) in a humidified chamber at 4C. Sections were covered with biotinylated secondary antibody for 30 min and then washed in PBS. Then, a peroxidase-labeled avidin/biotin solution reaction was applied. Slides were counterstained with hematoxylin. Subsequently, the sections were washed, dehydrated, mounted, and examined.

The sections stained with cleaved caspase 3 were examined at a magnification (×100) and the reaction was demonstrated in the cytoplasm of cells as brown coloration and evaluated subjectively according to the positive surface area, while the Immunohistochemical stained sections with Anti TNF were examined at a magnification (×400) and evaluated subjectively according to the staining intensity of the cytoplasm.

RESULTS

Light Microscopic Examination

Examination of left ventricles of control group showed that the cardiac muscle fibers appeared longitudinal arranged, branching, cylinders, of uniform diameters. The cardiac myocytes have acidophilic sarcoplasm with faint cross striations and central elongated vesicular nuclei. The muscle fibers appeared joined end to end with intercalated disc (Figures 1A-D).

Histo-pathological examination of ischemia-reperfusion group showed massive myocardial destruction and infarction which was widespread throughout the left ventricle and interventricular septum (Figure 2A). Large necrotic patches along myocardial tissue with interstitial edema and fibrosis, massive hemorrhage and diffuse polymorphic nuclear leucocytic infiltrate were observed (Figures 2B,C). Areas of fibrillolysis (hypokinetic defibrillated myofibers) and vacuolation of cardiac myocytes was noticed in most of examined fields (Figure 2D). Also, degeneration and fragmentation of cardiomyocytes, and vascular injury in the form of; swelling of endothelial lining, karyolitic nuclei and perivascular mast cell infiltration were seen (Figures 2E,F).

Examination of cardiac muscles of group III (I/R + Ptx group) showed improvement of histo-pathological features in most of examined sections throughout the left ventricle (Figure 3). Only sporadic areas of mild cardiac myocytes disarray, small focal area of muscle necrosis and perivascular fibrosis, minimal interstitial edema and hemorrhage (Figures 3A,B), and increased number of interstitial stellate cell deposition were observed (Figure 3C). Also, sporadic vacuolization of myofibers was present (Figure 3D). semithin sections showed well organized cross section of cardiac myocytes with remarkable increased number of blood capillary (Figures 3E,F).

Electron Microscopic Examination

Examination of left ventricle of group I showed striated cardiac myocytes with alternating dark and light band of myofibril and intact mitochondria arranged in rows parallel to myo-filament and condensed in the peri-nuclear region (Figures 4A,B). Examination of left ventricle, of Ischemia-reperfusion group, revealed fragmentation of myofibril and loss of

characteristic cardiac striation. The nucleus became large and rounded with dispersed chromatin. There was apparent decrease in the number of mitochondria which showed dispersion without peri-nuclear condensation. Moreover some of them showed destruction and fragmentation (Figures 4C,D). Wide interstitium with scattered extra-vasated RBCs, congested blood capillaries, lymphocytes and collagen fiber deposition (Figure 4E). The capillary endothelial lining was vacuolated, their nuclei show karyolysis, and presence of inflammatory cells adherent to the luminal surface of the endothelial cells (Figure 4F).

While, the examination of group III (I/R+ Ptx) revealed marked improvement of the myofibril and increased number of blood capillary scattered between the myofibril (Figure 5A). Restoration of the normal striation of cardiac myofibril (alternative dark and light band was obvious), convoluted nucleus indicating contractility and presence of condensed mitochondria (Figure 5B). Also, the endothelial lining of the capillaries appeared intact with no edema (Figure 5C).

Immunohistochemical evaluation

Examination of Immunohistochemical stained sections with cleaved caspase-3 revealed; positive reaction in a high proportional area of the wall of left ventricle in I/R group, as compared to the control. While, there were small sporadic positive area scattered in the wall of left ventricle of group III (I/R+ Ptx) as compared to control model (Figures 6A-C).

Examination of Anti TNF-α antibody stained section demonstrated marked strong positive reaction of cardiac myocytes of I/R group as compared to control one. While, weak reaction was detected in group III (I/RP+ Ptx), indicated by low intensity of brown color staining (Figures 7A-C).

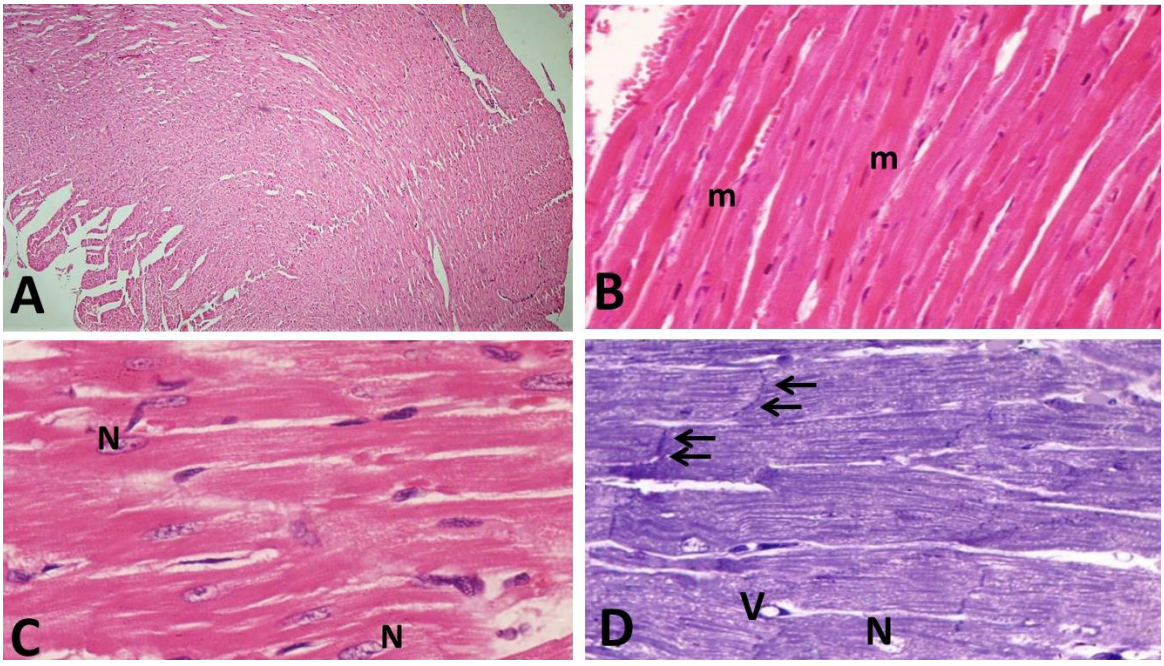

Fig. 1 [A-D]: Photomicrographs of sections of the Left ventricle of the control group. [A] Normal histological architecture of cardiac muscle. [B, C] Branching and anastomosing cylinder cardiac muscle fibers (m) with central elongated vesicular nuclei (N). [D] Cardiac myocytes anastomosing together at intercalated disc (→) and blood capillary (V). ([A-C] H&E stain and [D] toluidine blue stain, original magnification: x40 [A], x100 [B], and x400 [C-D])

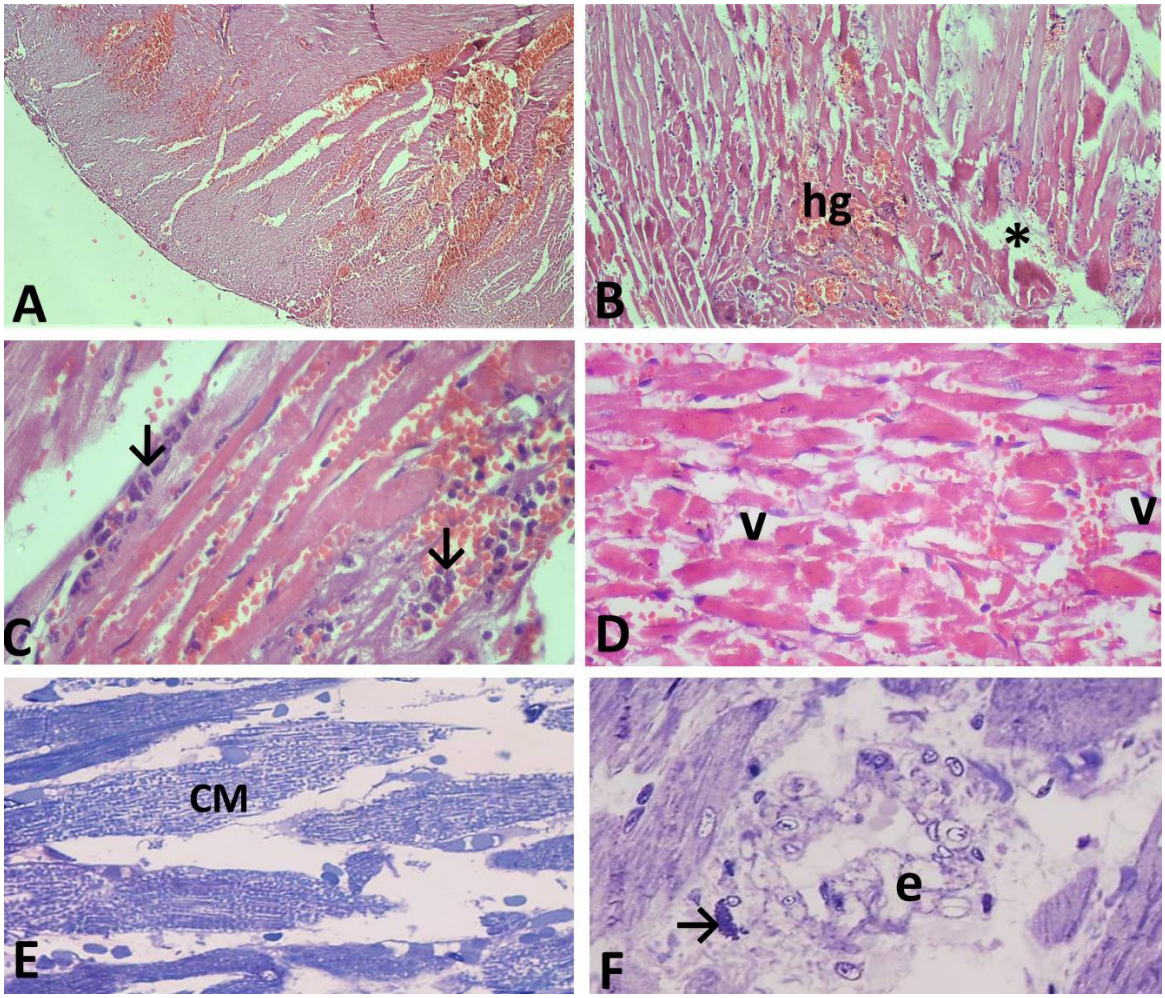

Fig. 2 [A-F]: Photomicrographs of sections of left ventricle of ischemic reperfusion group. [A] Massive hemorrhage in the ventricular wall. [B] Marked destruction of normal architecture of cardiac muscle, hemorrhage (hg) and necrosis (asterisk). [C] Necrosis of cardiac myocytes and polymorphic nuclear leucocytic infiltration (↓) in between. [D] fibrolysis and Vacuolation (v) of cardiac myocytes. [E] degeneration and fragmentation of cardiomyocytes (CM). [F] Vascular injury, endothelial swelling (e) and mast cell infiltration (→). ([A-D] H&E stain and [E-F] toluidine blue stain, original magnification: x40 [A], x100 [B], and x400 [C-F])

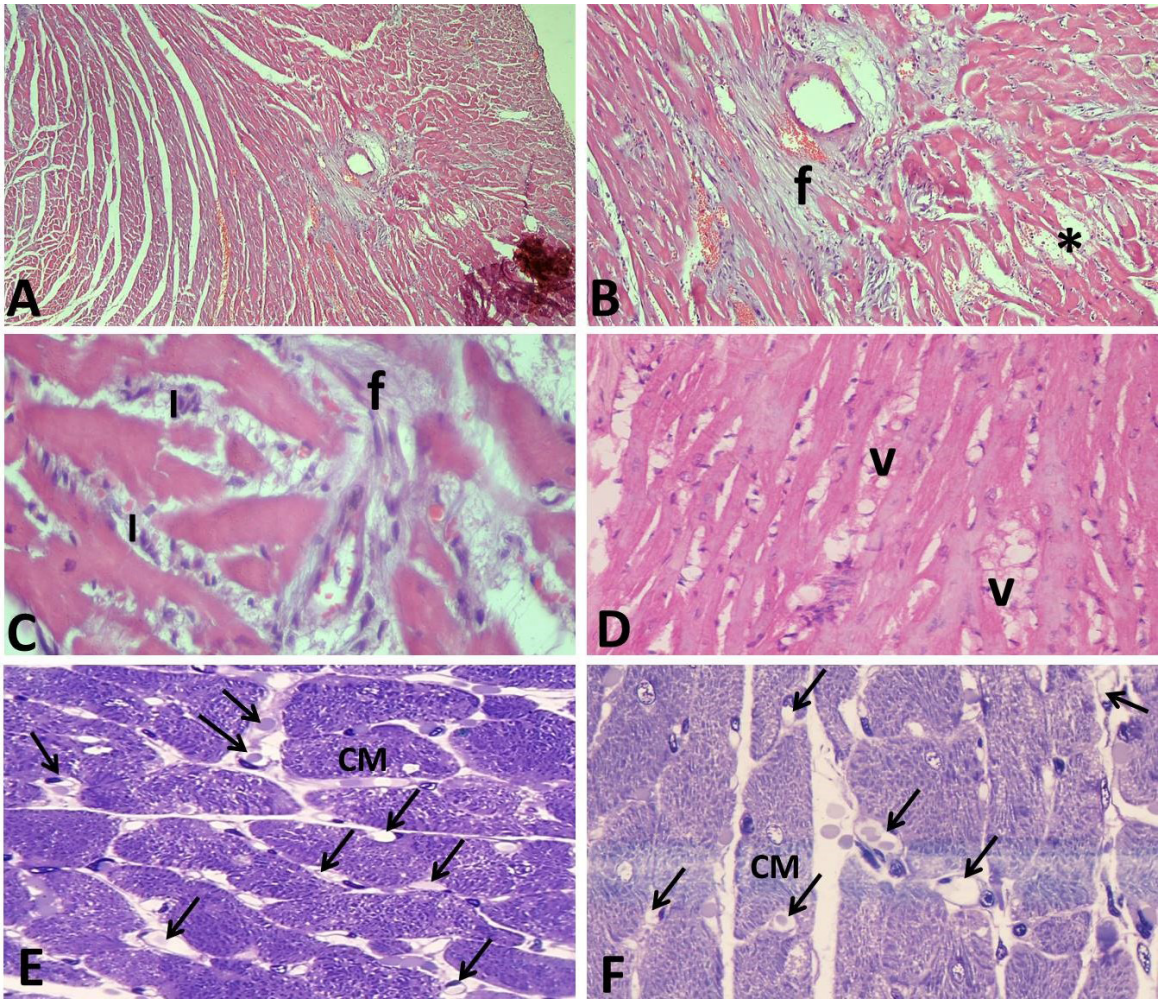

Fig. 3 [A-F]: Photomicrographs of sections of left ventricle of group III (I/R + pentoxiphilline). [A] Remarkable improvement of the histological picture of cardiac muscle. [B] Higher magnification of A, show sporadic area of necrosis (asterisk), mild hemorrhage and fibrosis (f). [C] Minimal fibrous tissue deposition (f) between cardiomyocytes with increased number interstitial stellate cells (I). [D] Sporadic myofiber vacuolization (v). [E, F] remarkable increased number of blood capillary (↓) between cardiac myocytes (CM). ([A-D] H&E stain and [E, F] toluidine blue stain, original magnification: x40 [A], x100 [B], and x400 [C-F])

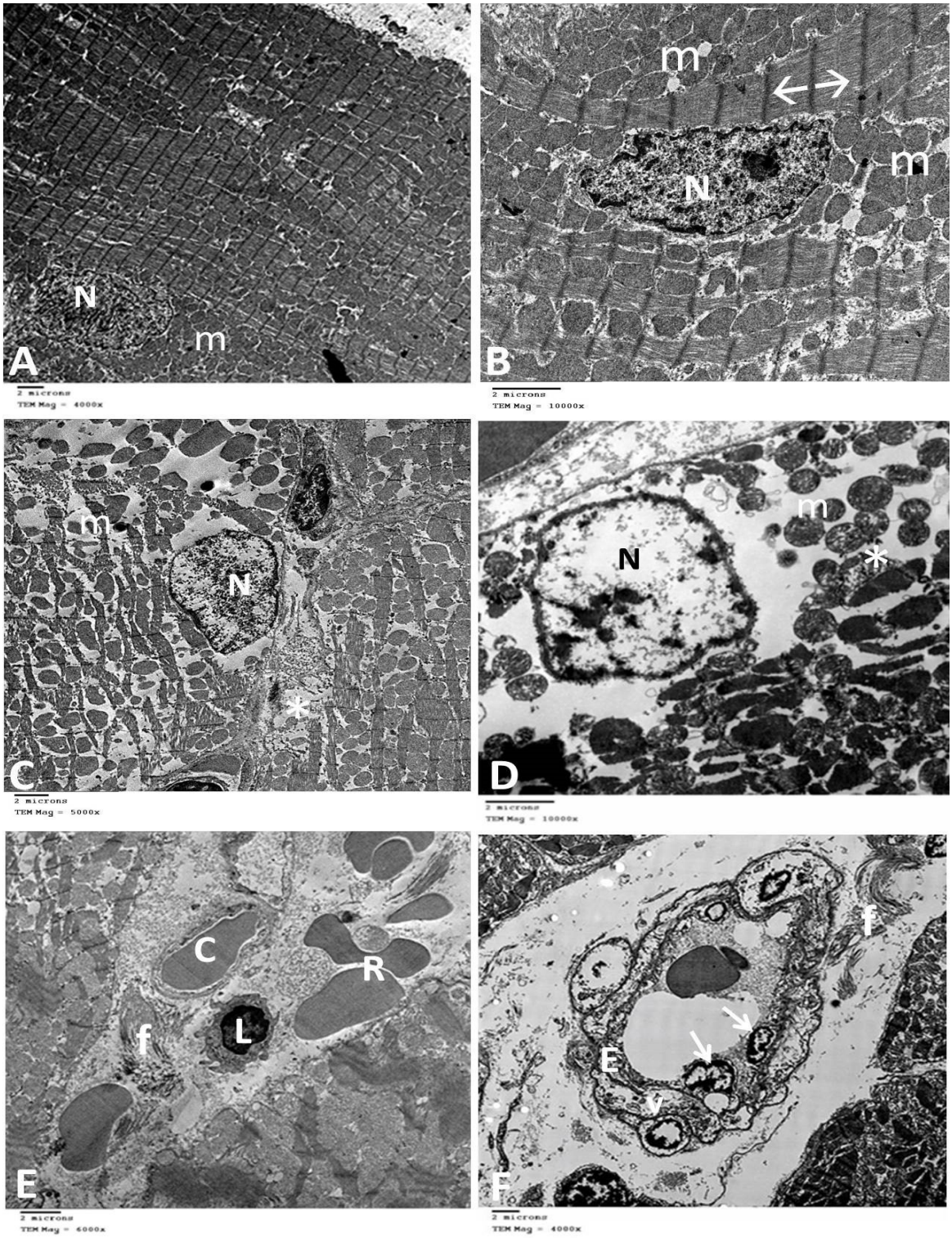

Fig. 4 [A-F]: Transmission electron micrographs of sections of left ventricle tissues. [A,B] control group shows regular striated cardiac myocytes alternative dark and light band (←→) with oval euchromatic nuclei (N), intact mitochondria(m) arranged parallel to muscle filaments and condensed in perinuclear space. [C-F] ischemic reperfusion group shows marked disturbed architecture, destructed myofibril, large round nucleus (N) with dispersed chromatin, less condensed mitochondria (m) and some are fragmented (*) , congested blood capillary (C), extravasated RBCs (R), collagen fiber deposition (f), lymphocytic infiltration (L), endothelial lining (E) of blood capillaries showing vaculated cytoplasm (V) and karyolitic nucleus, together with some inflammatory cells adherent to luminal surface (↓).

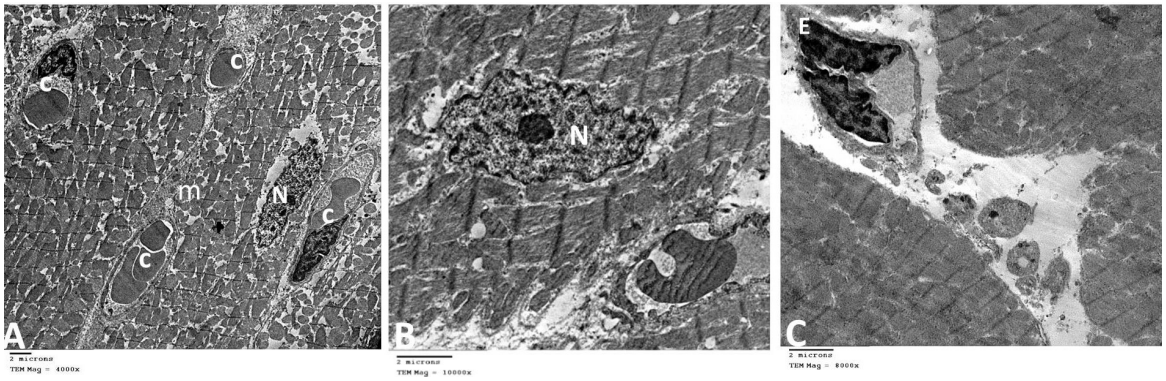

Fig. 5 [A-C]: Transmission electron micrographs of sections of left ventricle tissues of group III (I/R + pentoxiyphilline) show restoration of the normal striation of cardiac myofibril, convoluted nucleus (N), increased number of scattered blood capillary (C), condensed mitochondria (m), and normal endothelial lining (E) of the capillaries with no vacuolation.

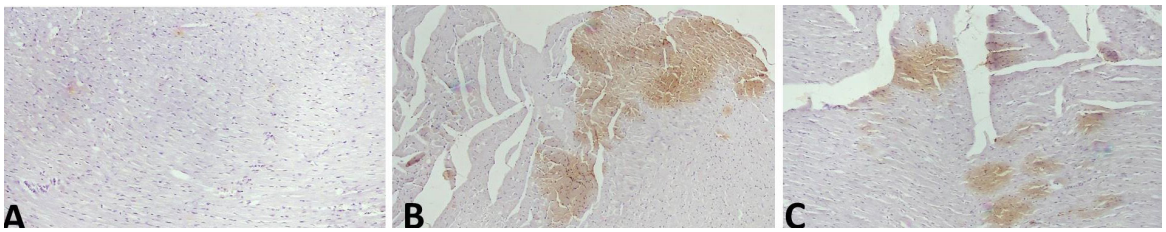

Fig. 6 [A-C]: Photomicrographs of sections of left ventricle stained with Caspase-3 immunohistochemical stain. [A]: no reaction in control group. [B]: I/R group show positive reaction in a large sector of myocardium. [C]: group III (I/R + pentoxiyphilline) show positive reaction in small sporadic foci scattered in the myocardium. (Magnification: X 100).

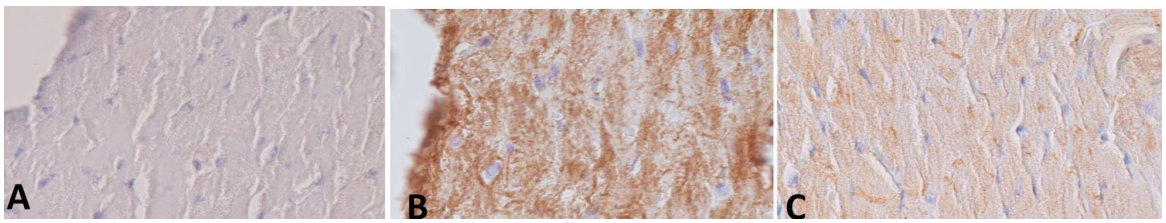

Fig. 7 [A-C]: Photomicrographs of sections of left ventricular stained with Anti TNF-α immunohistochemical stain. [A]: control group show no reaction. [B]: I/R group show strong intense reaction of cardiac myocytes. [C]: group III (I/R + pentoxiyphilline) show weak reaction of cardiac myocytes. (Magnification: X 400).

DISCUSSION

There are a number of factors to be considered before any successfully treatment of ischemic injury in the heart. It has been reported that timely reperfusion through percutaneous coronary intervention is considered the most effective treatment for preserving ventricular function, reducing infarction size, and preventing heart failure[4]. Although, the process of restoring blood flow in coronaries is a mandatory process requested for rescue viable cardiac myocytes, reperfusion can paradoxically injury the myocardium and result in cell death.

The underlying pathophysiological mechanisms of the process of Ischemic reperfusion injury (IR) are not yet completely understood. Several pathways have been proposed, including acute inflammatory reaction, release of reactive oxygen species (ROS), intracellular Ca2+ overload, depletion of ATP, and impaired metabolism[22]. These alterations may collaboratively act and produce irreversible damage to ischemic re-perfused cardio-myocytes and Cell death.

In the present study, marked destruction of cardiac architecture, necrosis, muscle lysis and fragmentation, massive hemorrhage, polymorphic nuclear infiltration, vasculopathy in the form of swelling of endothelial cells, leukocyte endothelial adhesion, accumulation of neutrophil, and vascular congestion, were identified in most of examined section of cardiac muscle of ischemic/reperfusion group. These findings are in

agreement with previous studies which illustrated the morphologic changes of cardiomyocyte in the ischemic-reperfused myocardium[23,24]. Our work demonstrated that administration of Ptx immediately before reperfusion extensively diminished the degree of muscle necrosis and provided greater protection against myocardium IR induced cardiac injury. This was in agreement with Adams *et al*.[25], who reported that Ptx reduced the extent of skeletal muscle damage induced by IR injury.

In addition, administration of Ptx showed reduction of fibrous tissue deposition in between muscle fibers. Previous studies reported that Ptx can decrease the level of fibrinogen by increasing fibrinolytic activity or reducing fibrinogen production in patients with peripheral vascular disease[26,27].

Remarkable apparent increase in number of blood capillaries was observed in group received Ptx. Also, there was great improvement in the structure of endothelial lining of the blood capillary. So, our study clarified the protective role of Ptx on blood vessels and its promotive action in enhancing new angiogenesis.

Several investigations have suggested that the effectiveness of Ptx stems from its hemorheological property and other pharmacological actions, such as the reduction of blood viscosity and enhancement of fibrinolytic activity[28]. Furthermore, Ptx can improve the permeability of blood vessels and prevent endothelial injury through scavenge hydroxyl radical and reactive oxygen species[29,30] and also protects mitochondrial structures[31]. Most previous experiments demonstrated the vasodilation effect of Ptx on vascular bed of the skeletal muscl[e][13]. Thus, Ptx is considered to be of great potential for treating various circulatory disorders clinically.

In the present study, there was marked infiltration of the ischemic cardiac tissue by different types of inflammatory cells, especially neutrophils and lymphocytes. While, administration of Ptx before induction of I/R injury, showed apparent decrease in inflammatory cells accumulation inside the injured myocardium. These finding could be explained according to previous investigations which reported increased concentrations of chemotactic mediators as polypeptide and others immune complexes factors after reperfusion of the ischemic tissue[32,33]. These mediators attract monocytes, poly-morphonuclear leucocytes (PNL), and macrophages to the environment. Accumulation PNL in the tissue will initiate the tissue damage by triggering several reactions[34] including increased endothelial damage and permeability, secretion of collagenases and elastases.

Because neutrophil adhesion plays a key role in ischemia-reperfusion injury, finding a way to block this adhesion is considered an important step in establishing a treatment for these injuries. Incidentally, it has been reported that Ptx reduced the damage to the tissue through preventing the accumulation of the cells and modulating the cytoskeletal interactions[35-37].

TNFα (Tumor Necrosis Factor alpha) is a proinflammatory cytokine superfamily which is produced by lymphocytes (natural killer), macrophages and fibroblasts. they have a great potential role in the evolution of inflammation. It has been implicated in a variety of biological processes including cell proliferation, differentiation, and apoptosis. Also, it is involved in a wide spectrum of autoimmune diseases[38].

Our results demonstrated that there was accumulation of TNF-α in the myocardium of ischemic reperfusion group, which was cleared by strong positive intense reaction of cardiac myocytes after immunohistochemical staining with anti TNF-α antibody. These finding are in agreement with previous studies which reported that accumulation of TNF-α and IL-1 within ischemic tissue directly injures the tissue and leads to the release of oxygen free radicals as ROS, which can inhibit cell proliferation, induce apoptosis and results in further damage[39-42].

On the other hand, administration of Ptx revealed marked reduction of TNF-α expression. This finding is supported by the hypothesis that Ptx decreases the inflammatory response of myocardium through reducing the production of TNF-α and other inflammatory mediator and provides lasting power to myocardium,[43,13] Growing evidence suggests that myocardial ischemia-reperfusion (IR) produces cardiac dysfunction and Cell death through exacerbation of both cardiomyocyte apoptosis and necrosis[44,45]. Previous study reported the effective prominent role of activated caspase-3 in the execution phase of apoptosis by controlling DNA fragmentation[46].

Therefore, in the present work, myocardial sections were immunohistochemical stained with cleaved (activated) caspase-3 for detection of apoptosis. Positive expression of cardiac

myocytes was detected in most of examined sections, indicating active process of apoptosis induced by IR injury. While, marked reduction in expression of caspas 3 was present in group III (IR+Ptx). These findings clarify the cardio-protective effect of Ptx and demonstrate its anti-apoptotic effects.

CONCLUSION

Thus, the current study has demonstrated the potential effect of Ptx in preventing I/R injury and protecting cardiomyocyte from cell death. This could be achieved through inhibition of inflammation, prevention of apoptosis, improving cardiac hemodynamics, and preservation of myocardial morphology. We concluded that Ptx has great potential as a therapeutic intervention for helping patients recover from clinically common ischemia-reperfusion injuries.

CONFLICT OF INTERESTS

There are no conflicts of interest.

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التاثير الوقائى المحتمل لدواء البنتوكسيفيلين على إصابة عضلة القلب الناتجة عن قطع الدم وإعادة الترويه في نموذج الجرذ : دراسة هستولوجية وهستوكيميائية مناعية

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ملخص البحث

المقدمة: على الرغم من أن عمليه إعادة ضخ الدم هي الطريقة المثلى المطلوبه لإنقاذ عضلة القلب وابقائها علي قيد للحياة، الا ان عملية استعادة تدفق الدم فى الشرايين التاجيه قد تؤدى الى تغييرات عكسيه ينتج عنها موت خلايا عضلة القلب٠ ويجري حاليا عدد من الاستراتيجيات العلاجية الجديدة تهدف الى منع إصابة عضلة القلب بالاحتشاء الدموى وتحسين النتائج.

الهدف من البحث: اقيمت هذه الدراسه لتقييم الفائدة المحتملة من استخدام البنتوكسيفيللن فى تقليص الاصابة فى عضلة القلب الناتجة عن قطع الدم وإعادة الترويه.

المادة و طرق البحث: تم استخدام ثلاثين جرذ من ذكور الجرذان البيضاء وتقسيمهم الـ ثلاثه مجموعات تشمل كل مجموعة عشر جرذان. المجموعة الضابطة، المجموعة الثانية الخاضعه لعمليه قطع الدم وإعادة الترويه حيث تم احداث انسداد مؤقت فى الشريان التاجي الامامى الهابط لمده ٤٥ دقيقه واعقبه اعاده ضخ الدم لمده ١٢٠ دقيقه، والمجموعة الثالثة: تم حقن الجرذان داخل الصفاق بجرعة من عقار البنتوكسيفيللن مره واحده ١٥ دقيقه قبل احداث عمليه قطع الدم واعاده الترويه٠ عند انتهاء وقت التحربه، تم جمع عينات من البطين الايسر من حميع الجرذان وتم تمريرها ومعالجتها ثم فحصها بواسطه المجهر الضوئى والمجهرالإلكتروني. ايضا تم صباغه بعض الشرائح بالصبغات الهستوكيميائية المناعية لإظهار موت الخلايا المبرمج واظهار مضادات الالتهابات.

النتائج: لقد أظهر الفحص الهستولوجى للانسجه ان المعالجة المسبقة باعطاء البنتوكسيفيللين قد ادت الى حمايه عضله القلب من الاصابه الناتجة عن قطع الدم وإعادة الترويه وقد اتضح ذلك من خلال التحسن الظاهر فى التركيب النسيجى لعضله القلب، ظهور كميه قليله جدا من الارتشاح والنزيف و التليف. عدم وجود تجمعات لخلايا الالتهاب. كما وجد انخفاض ملحوظ فى نسبه موت الخلايا فى عضله القلب مما يشير إلى قوه التأثير المضاد للبنتوكسيفيللين على موت الخلايا المبرمج، و قد ظهرت ايضا قوه تأثير للبنتوكسيفيللين كمضاد لاالتهابات.