RESEARCH ARTICLE

CORM2 protects from myocardial ischemia reperfusion injury via modulation of the inflammatory response and apoptosis

Najah R Hadi*
Fadhil G Al-Amran[1]
Karrar A Muhsin[2]
Abbas Taher[3]

*Dept of Pharmacology and Therapeutics, Faculty of Medicine, University of Kufa, Kufa, Iraq
[1]Department of Surgery, Cariothorasic Section, Faculty of Medicine, University of Kufa, Kufa, Iraq.
[2]Dept of Pharmacology and Therapeutics, Faculty of Medicine, University of Kufa, Kufa, Iraq
[3]Department of OMF Surgery, Dean of Faculty of Dentistry, University of Kufa, Kufa, Iraq

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ABSTRACT

Background: Myocardial ischemia is one of the major clinical problems in the world. There are two forms of the cell injury occur during myocardial ischemia, which are necrosis and apoptosis. Reperfusion is very important to maintain the viability of the myocardial cells, but in the other hand reperfusion not free from hazardous effects and it is usually associated with what is called ischemia reperfusion injury.
Objective: This study was undertaken to assess the possible protective potential of Carbone monoxide releasing molecule-2 (CORM2) in regional myocardial ischemia reperfusion injury and apoptosis in male rats.
Methods: Twenty four adult male Swiss albino rats were divided into four groups (six rats per group). Sham , in a uniform manner, surgical procedure was done for the rats in all groups, and in this group there was no ligation for (LAD). Control group, rats were subjected for ligation of LAD for 30minutes then 3hr reperfusion. vehicle group, rats were subjected for ligation of LAD for 30minutes then 3hr reperfusion and received 0.5%DMSO before reperfusion time. Treatment group: reperfusion lasted for 3 hours after LAD ligation for half an hour, treatment with CORM2 at reperfusion time (8mg/kg I.V via the tail vein) have done to all rats.
Results: At the end of reperfusion animals sacrificed and cardiac TNF-α, IL-1β ,IL-6, ssDNA and plasma troponin I (cTnI) were measured. It has been found that CORM2 treated group showed a significant reduction ($P< 0.05$) in cardiac TNF-α, IL-1β, IL-6, ssDNA and plasma cardiac troponin I (cTnI) compared with the control group. Histopathological study found that treatment with CORM2 significantly ($P<0.05$) reduce the myocardial injury compared with the control group
Conclusion: CORM2 lead to reduction in regional myocardial ischemia reperfusion injury and apoptosis during ischemia via interfering with inflammatory pathway.

Keywords: CORM2, Myocardial ischemia, Reperfusion

Introduction

A major cause of death in the world is the coronary artery diseases. More than 6 millions people die each year because of it. Re-introduction blood into an ischemic organ needed to inhibit cellular loss, but this can induce injury. This phenomenon is termed myocardial reperfusion injury (1). Ischemia reperfusion causes local cellular hypoxia that is accompanied by inflammatory responses that lead to the recruitment of leukocyte and subsequent damage (2). Ischemia has been shown to lead to endothelial dysfunction with an increase in permeability, an increase expression of adhesion molecule and recruitment of leukocyte (3) During ischemia there will be a process of catabolism for adenine nucleotide, result in accumulation of hypoxanthine,reintroduction of oxygen and (ROS) formation. Which will upregulates the gene expression for many inflammatory agents (e.g.,leukocyte adhesion molecule and cytokines) and bioactive agent (e,g.,endothiline,thromboxaneA2)while repressing other "protective"gene products (e.g.,constitutive nitric oxide synthase ,thrombomoduline)and bioactive agent (e,g.,prostacycline ,nitric oxide)(4). Prolonged periods of myocardial ischemia are related to an increase in the rate of necrosis, whereas, paradoxically, reperfusion leads to an augmentation in apoptosis(5,6). has raised particular therapeutic interest because of its potent The antiinflammatory and anti-oxidant activity for Carbon monoxide (CO) gives it a good attention regarding its use for therapeutic purposes, although it's a toxic gas that have the ability to impaire the respiratory system. However, CO is also produced by the protein heme oxygenase (HO) and as such functions as a potent endogenous antioxidant that counteracts toxic effects of ROS (7). CO inhibited the production of proinflammatory cytokines, such as TNF-α, MIF and interleukin-1, from macrophages (8). Carbone monoxide stimulated the synthesis of the anti inflammatory cytokine interleukin-10 (8). Exposure of macrophages to a low concentration

Address for correspondence

Dr. Abbas Taher
Faculty of Dentistry, University of Kufa, Kufa, Iraq
Email: kufa54@gmail.com

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of CO (250 ppm) as well as over expression of HO-1 in these cells inhibited lipopolysaccharide- induced production of granulocyte macrophage colony-stimulating factor (GM-CSF). This effect of CO was mediated by inhibition of the transcription factor NF-κB (9-11). Carbone monoxide-donor pretreatment activated progenitor cell and promoted vasculogenesis and the formation of new cardiomyocytes after myocardial infarction in rats(12). The HO-1 gene transfer has earlier been shown to protect against angiotensin II-induced cardiomyocyte apoptosis in vitro and against ischemia / reperfusion (I /R) injury and cardiomyocyte apoptosis after repeated I/R episodes in rat hearts in vivo (13).

Methods:

Material

CORM2 powder (pure) from (Sigma, USA), Xylazine 2% vials (RompunTM, Bayer AG, Leverkusen, Germany), ketamine (Hikma, Jordan), ethanol (Fluka, Switzerland) and normal saline (KSA). Rat (IL-1β), (IL-6), (TNF-α) ELISA kits (Sigma, USA). Rat cTnI ELISA (Life diagnostics Inc., USA). High Intensity Ultrasonic Liquid Processor (Sonics & materials Inc., USA), Vascular Clamp (Biotechno, Germany) and ventilator (Harvard. USA).

Animal

Male swiss albino rats and their weight was between (180-220 g) were purchased from Animal Resource Center , National Center for Drug Control and Research. The rats kept at 25˚C and 12-hour light-dark cycle for two weeks with free access for water and food. Animals had no manifestation of any illness upon examination.

Study design

Rats were randomized into 4 groups 6 animals in each group as follow:

1. group 1 (Sham) : the same anesthetic and surgical procedures applied to rats of this group without ischemia.

2. group 2 (Control) : (induced untreated group): Rats underwent 30 min of LAD ligation followed by a 3hr of reperfusion.

3. group 3 (vehicle) : Rats underwent 30 min of LAD ligation and 3hr reperfusion and received 0.5%DMSO before reperfusion time.

4. group 4 (CORM2 pre-treated) : in this group, all rats undergo 30 min of ischemia and 3hr reperfusion with CORM2 at reperfusion time was given (8mg/kg i.v via the tail vein) .

Stock powder dissolved in100% DMSO(dimethylsulfoxide) which then diluted to 10% with saline to invivo use. It was prepared immediately before use.(14,15)

Surgical LAD ligation

The rats were anesthetized by intraperitoneal injection with a mixture of ketamine and xylazine in a dose of 100mg/kg and 10 mg/kg respectively (16). The trachea was intubated with a cannula and ventilation was achieved by connecting the tube in the trachea to the ventilator supplied with 100% oxygen at a respiratory rate of 50/min with a tidal volume of 20 ml/kg body weight (17). Animal ventilated using a small animal ventilator (Harvard Apparatus, Holliston, MA, USA). Then a lateral thoracotomy approach is done , heart was exposed and left anterior descending branch of the left coronary artery was ligated, then chest was closed. The survival rate following the surgery was around 60%–70% and was equivalent between groups. After 30 min reopen the chest and the ligature removed at this point reperfusion started which is last for 3hrs.

Collection of Samples

At the end of reperfusion The blood was collected from the ventricular apical side. Hearts were cut from their main arteries and rinsed with normal saline to remove any debris, then stored in deep freeze at (-20˚C to -80˚C). The ventricles were cut from atrioventricular junction, each ventricle was divided into 2 parts, apical part and basal part. The apical part further more divide into two parts, one for measurement of apoptosis level and the other was fixed in 10% formalin and prepared for routine histological processing by embedding in paraffin blocks (18). To do histological cheking , 5µm- sections

were taken and stained with haematoxylin-eosin (H&E).

Samples preparation

1-Preparation of Sample for TNF-α and IL-1β and IL-6 measurements

Upper part of ventricles were rinsed with ice cold saline, then homogenized in phosphate buffered saline with a ratio of 1:10 (w/v) that contained protease inhibitor cocktail and 1% Triton X-100 with ultrasonic liquid processor **.** centrifugation for homogenate was done for 20 min at 4°C at 2,500 *g*. The supernatant was collected for determination of TNF-α, IL-1β, IL-6ELIZA kits(Sigma Aldrich, USA) (19).

2- cTn-I measurement

Blood was collected from the apex of each heart by a syringe needle inserted directly to the heart. EDTA (22 mg/ml) tubes was used for blood samples collection, mixed thoroughly and centrifuged at 3000 RPM for 15 min.

3-Detection of Apoptotic Cells by Foramamide

Formamide denaturation to DNA in apoptotic cells and not in necrotic cells or in the cells with DNA breaks in the absence of apoptosis was used in this assay that denatured DNA with monoclonal antibody to single-stranded DNA (ssDNA) was detected. The used tissue for measurement of apoptosis level were lysed using trypsin.

Statistical Analysis

By using SPSS 20.0 for windows, the analysis was done statistically. An expert statistical advice was consulted for tests used. Analysis of Variance (ANOVA) was used for the multiple comparisons among all groups followed by post-hoc tests using the LSD method. Changes in the histopathology differences in total score between more than 2 groups was assessed by Kruskal-Wallis test, while Mann-Whitney U test was used for the difference between 2 groups. In all tests, $P< 0.05$ was considered to be statistically significant.

RESULTS

1- Effect of Carbone monoxide releasing molecule-2 on the proinflammatory marker (TNF-α, IL-1β and IL-6)

At the end of the experiment and in the control group ; the levels of cardiac (TNF-α, IL-1β and IL-6) was increased significantly ($P<0.05$) compared to sham group.

The levels of cardiac (TNF-α, IL-1β and IL-6) of CORM2 treated group was significantly lower($p<0.05$) than both control and vehicle groups. (Figure 1)

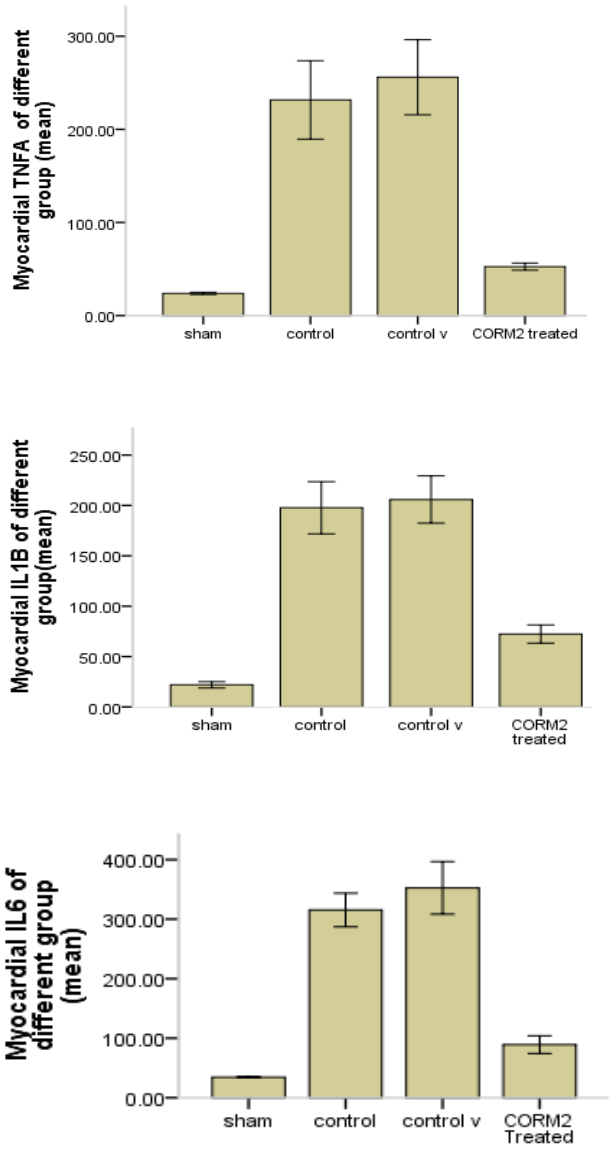

Figure 1: The means of cardiac (TNF-α,IL-1βand IL-6) levels (pg/mg) were found to be significantly elevated ($P<0.05$) in the control group and control vehicle compared with sham group . At the same time, cardiac cytokines were significantly decreased ($P<0.05$) in CORM2 treated group with respect to both control and control vehicle groups.

2-The effect of Carbone monoxide releasing molecule-2 on the cardiac troponin I

At the end of the experiment, the level of plasma (cTnI) was significantly increased (P<0.05) in the control group as compared with the sham group.

The plasma level of (cTnI) of CORM2 treated group was significantly (p<0.05) lower than that of control and control vehicle groups. (Figure 2)

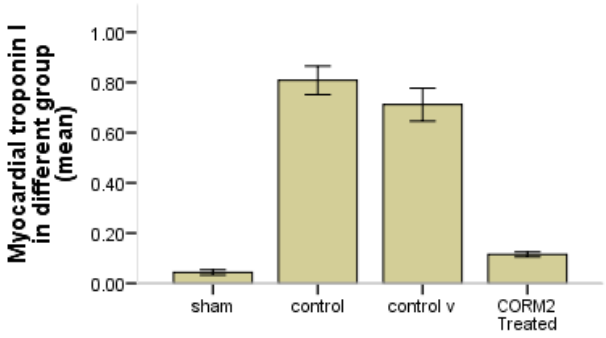

Figure 2: The level of plasma cTnI (ng/ml) was significantly increased (P<0.05) in the control group and control vehicle compared to the sham group . On the other hand, cTn-I was significantly reduced (P<0.05) in CORM2 treated group with respect to both control or control vehicle groups.

3-Effect of Carbone monoxide releasing molecule-2 on the level of apoptotic cell

At the end of the experiment, the result shows that there is a significant increase (p<0.05) in apoptotic cell in control, control v compared with the sham group. CORM2 group show significant decrease (p<0.05) in apoptotic cell compared with control and control vehicle groups. CORM2 treated group showed a significant increase (p<0.05) compared to the sham group. (Figure 3)

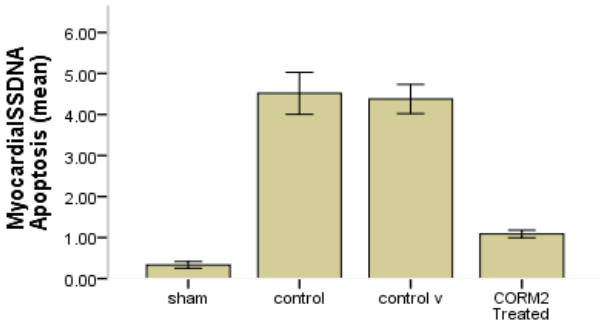

Figure 3: The mean of cardiac apoptosis level

4-Effect of Carbone monoxide releasing molecule-2 on the histopathological score

CORM2 treatment significantlyi mproved cardiac injury (P < 0.05) compared to control group and the total severity scores mean of this group showed 33.3% of the group had no damage, 66.7% mild injury. Sham heart tissue showed a normal cardiac structure. All rats in this group showed normal hearts 100%. There was statistically insignificant difference between control vehicle group and control group (P=0.685) and the total severity scores showed 33.3% of the groups had severe cardiac injury and 66.7%had highly severe cardiac injury (Figure 4, 5)

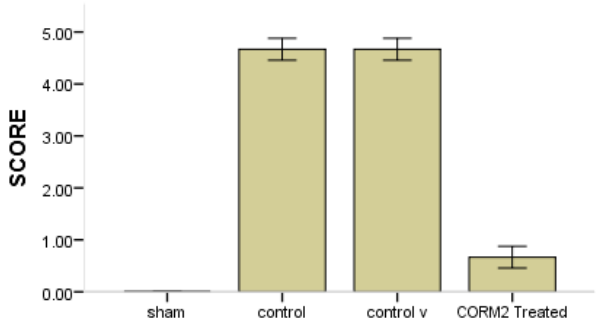

Figure 4: Total myocardial damage scores

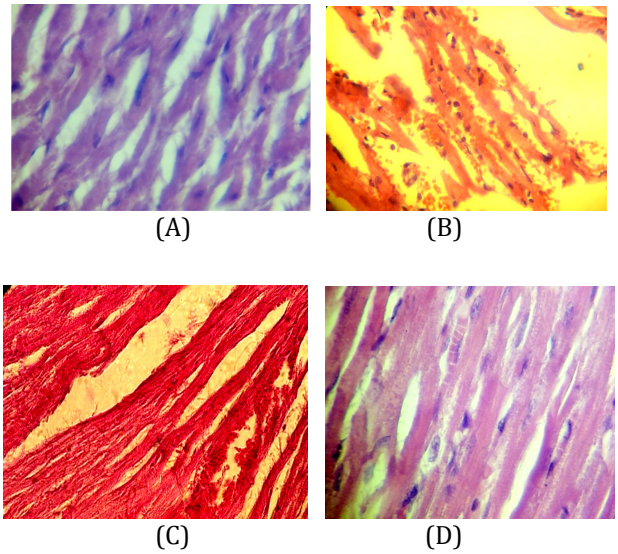

Figure (5): (A) cardiac section of normal rats with normal architecture. The section stained with Haematoxylin and Eosin(X40)**(B)** cardiac section showed interstitial edema, focal necrosis , contraction band,PMN infiltration, highly sever injury . The section stained with Haematoxylin and Eosin(**C**) cardiac section showed capillary compression sever injury.(**D**) cardiac section treated with CORM2. Haematoxylin and Eosin was used as staining agents (X 40).

Discussion

Myocardial damage resulting from ischemia-reperfusion (I/R) is a major cause of morbidity and mortality in the world, those myocardial I/R injuries result in cardiac dysfunction, arrhythmias, as well as irreversible myocyte

damages (20). Inflammatory responses have role in I/R injury mediated by activation of cytokines and adhesion molecules (21). Inflammatory cytokines, such as TNF-α, IL-1β and IL-6 were shown to play key roles in the pathophysiology of ischemia and reperfusion injury (22). Many experimental studies have shown the specific and independent role of exogenous CO in the modulation of inflammation (23, 24). The existence of compounds based on metal carbonyl (CORMs) that control the CO releases in biological systems gives more opportunity to investigate CO-mediated biological effects than before (25). The vaso-active, antihypertensive, cardio protective and anti-rejection effects of CORM-released CO in vivo have been demonstrated (11, 26-30). In the present study, CORM2 significantly reduced the elevation of inflammatory mediators (TNF-α, IL-1β And IL-6) levels in cardiac tissues ($p<0.05$) compared to control group, gives orientation about protective effect of CORM2 in myocardial I/R injury. In our knowledge, there is no study on CORM2 and (TNF-α, IL-1β And IL-6) However, Sawle et al. (2005) show that CORM-2, a DMSO-soluble CO-RM, gives an anti-inflammatory effect in an *in vitro* model of LPS-stimulated macrophages (31). Bingwei et al. (2006) show that *In vivo* application of CORM-2 in burn mice markedly decreased the production of IL-1β and TNF-α in BAL fluid.(32) CORM2 treatment rats shows a significant reduction in cardiac injury ($P < 0.05$) compared to control group and the total severity scores mean of this group showed mild cardiac injury,also there is no study about the effect of CORM2 on histopathological score. Bingwei et al.(2006) shows that PMN chemotaxis and infiltration in the lung after thermal injury effectively prevents by the use of CORM-2, with decrease in the production of oxidants and reduction in tissue oxidative injury. CORM-2 usage in a model of burn mice reduces the accumulation of PMNs , inhibits NF-κB activity and decreases the production of inflammatory mediators.(32) Yunwei et al. (2010) show that CORM2 can decrease the total severity score of the histopathology by the decrease of PMN cell as neutrophil in treated hepatic I/R injured rat.(14)

In the present study, we found that CORM2 treatment significantly ($P < 0.05$) reduced the increasing plasma levels of cTnI as compared with the control group. According to our knowledge; there are no data available about effect of CORM2 treatment on cTnI. However Atsunori et al.,(2010) find that CO treatment with hydrogen decrease significantly cardiac troponin I in the plasma (33).

Treatment of rats with CORM2 reduces apoptotic cell significantly ($P < 0.05$) as compared with the control group. Gunther et al., (2002) show that the cytoprotective effects of CO have also been associated with inhibition of apoptosis and up regulation of antiapoptotic proteins (34). Guangwu et al., (2010) proved that CO delivery exogenously in an *in vivo* by CORM-3 improved post infarction LV remodeling and dysfunction and reduced myocardial apoptosis (35). Recently Zrelli et al.,(2013) that CORM2 synergistically strengthen the antiapoptotic effects of hydroxylthirosol via suppression of caspase-3 activation (36).

Limitation of the study

TUNEL technique was not used in this study to differentiate between apoptotic and necrotic neuclei .

Conclusions:

We conclude that CORM2 has a cardioprotective potential as it ameliorates myocardial ischemia reperfusion injury via interfering with inflammatory responses and apoptosis.

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