INVOLVEMENT OF NITRIC OXIDE DEPENDENT MECHANISM IN APELIN ACTION ON MYOCARDIAL ISCHEMIA/REPERFUSION INJURY IN MALE ALBINO RATS

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ABSTRACT

Background: Apelin acts as a regulating peptide of cardiovascular, hypothalamus, hypophysis, metabolic, gastrointestinal and immune systems. **Objective:** The goal of this study was to clarify whether apelin-13 is cardioprotective against ischemia/reperfusion injury if given as either a pre- or postconditioning mimetic and whether nitric oxide (NO) is involved in apelin-induced protection. **Design:** Langendorff perfused rat hearts underwent 30 min of global ischemia and 120 min of reperfusion. Infarct size and lactate dehydrogenase, creatine kinase –MB and malondialdehyde release were determined to evaluate the severity of myocardial injury. Apelin-13 was infused at 0.5 µM concentration for 20 min either before ischemia or in early reperfusion, without and with NO synthase inhibition by NG-nitro-L-arginine –methyl ester (L-NAME). In additional experiments, before ischemia also 1 µM apelin-13 was tested. **Results:** Whereas before ischemia apelin-13 (0.5 and 1.0 µM) was ineffective, after ischemia it reduced infarct size, lactate dehydrogenase, creatine kinase –MB and malondialdehyde release when compared with control group ($P<0.001$). In addition, it could be noticed that the cardioprotective effect of apelin was abolished in the presence of L-NAME, a nonspecific NOS inhibitor. **Conclusion:** Apelin-13 protects the heart only if given after ischemia. In this protection NO plays an important role. Further studies are recommended on the endogenous origin and the mode of action of apelin-13 within the pool of treatments under study for postinfarction therapy as well as the use of markers for NO detection, and selective inhibitors of NOS for further delineation of apelin effects on ischemic myocardium.

Key words: Apelin, ischemia/ reperfusion injury, nitric oxide.

INTRODUCTION

Severe myocardial dysfunction and tissue damage resulting from ischemia/reperfusion (I/R) is a common clinical scenario in patients with some heart diseases and therapies such as thrombolysis, percutaneous coronary intervention, coronary artery bypass grafting, and cardiac transplantation. The phenomenon that reperfusion causes damage in addition to that caused by the ischemia insult is referred to as I/R injury [(1)]. Myocardial ischemia and subsequent reperfusion lead to formation of a number of intrinsic factors which mediate the cellular mechanisms of adaptation to altered oxygen and energy supply. One of them is adipocytokine apelin, which isolated from bovine stomach extracts and identified as the endogenous ligand of the human orphan G protein-coupled receptor APJ [(2)].

Apelin is the endogenous ligand for the G protein-coupled APJ receptor. Human, mouse, rat and cow apelin genes encode a 77-amino acid preprotein with the active sequence in the COOH-terminal region [(3)]. Various fragments of apelin have been isolated and classified according to the number of amino acids. Among fragments, apelin-13 and -36 are the most frequently studied, and apelin-13 is considered the most active of them [(4)]. Apelin receptors APJ have been found to be similar to angiotensin II receptor type 1 [(5)], but angiotensin II does not bind to APJ receptors [(6)]. Apelin mRNA is expressed in several organs and tissues. In the cardiovascular system, apelin and APJ receptors occur in vascular smooth muscle, endothelial cells, and cardiomyocytes [(7)]. Apelin exerts a positive inotropic effect [(8)] and a nitric oxide (NO)-driven vasodilator activity [(9)]. It protects the heart against ischemia/reperfusion (I/R) injury both in vitro and in vivo, but the underlying mechanism is still controversial [(10)]. In particular, a role of NO in mediating apelin-induced cardioprotection has not yet been defined [(4)].

Zeng et al. (2009) [(11)] observed antiapoptotic and antistunning activities by apelin administered in the same heart before and after global ischemia, a protocol that cannot clearly indicate the exact timing required to produce the protective effect. A remarkable protection against I/R injury can be induced with either ischemic preconditioning (preC) or postconditioning (postC). Whereas preC is obtained with one or more brief (a few min) coronary occlusions performed 5–10 min before the onset of ischemia, postC consists of very brief (a few seconds) occlusions carried out after the end of ischemia [(12)].

The goal of this study was to investigate the role of apelin on myocardial ischemia/reperfusion injury in male albino rats. In addition, the study was designed to clarify the involvement of NO-dependent mechanisms of apelin action on myocardial ischemia/reperfusion damage.

MATERIAL AND METHODS

Animals:

Six-month-old adult, male albino rats weighing 180-200 gm were used. The used rats were obtained from the animal house from faculty of veterinary medicine of Zagazig University. The animals were kept in steel wire cages (6-8/cage) in the physiology research laboratory and in animal

house in faculty of medicine of Zagazig University under hygienic conditions

Isolated Heart Preparation

Ten minutes after heparin (Nile CO.,Egypt) injection, animals were anesthetized with urethane (Prolabo,Paris) (1 g/kg) intraperitoneally. The hearts were rapidly excised and placed in ice-cold Krebs-Henseleit buffer (6.895 gm/L NaCl, 0.350 gm/L KCl, 0.280 gm/L CaCl2, 0.160 gm/L NaH2PO4, 0.290 gm/L MgSO4, 2.1 gm/L NaHCO3-, and 2.1 gm/L glucose). Then the hearts were suspended to a Langendorff apparatus (416-6122-Palmer, London) and retrogradely perfused via aorta at constant flow with the above Krebs-Henseleit buffer in a non recirculating way. The perfusate buffer was saturated with a 95% O2-5% CO2 gas mixture and infused at 37°C as previously described [13].

Experimental protocol:

In studies on myocardial protection by apelin, the apelin-13 fragment (Sigma-Aldrich Chemical, St. Louis, MO.) was chosen because it has been reported to exhibit the most potent effect on myocardial protection [4].

To find out the minimum dose of apelin-13 capable to reduce reperfusion injury, in previous experiments, the peptide was infused during the first 20 min of reperfusion at 0.1, 0.2, 0.5, and 1.0 µM concentrations. Because 0.1 and 0.2 µM were ineffective, the bulk of this study was performed at apelin concentrations of 0.5 and 1.0 µM. (n. 2 for each concentration) [12].

After 20 min of stabilization, each heart underwent ischemia/reperfusion (I/R) consisting of 30 min of global ischemia followed by 120 minutes of reperfusion. Hearts were randomly assigned to one of the following experimental protocols ***(Fig. 1):***

Group 1 (control; n.:10): these hearts underwent I/R without any addition.

Group 2**:** these hearts were divided into 2 sub groups:

Group 2 A (Ap-pre-0.5; n.:10): in this group hearts were perfused before ischemia with 0.5 µM concentration of apelin-13 for 20 min followed by 10 min of washout to mimic ischemic preconditioning, At the end of washout, I/R was performed.

Group 2 B (Ap-pre-1.0; n.:10): in the hearts of this group apelin-13 was given before ischemia as in group 2 A but at 1 µM concentration.

Group 3 (Ap-post-0.5; n.:10): after ischemia, these hearts were perfused with 0.5 µM concentration of apelin-13 during the initial 20 min of reperfusion to mimic ischemic post conditioning.

Group 4 (Ap-post-0.5 µM with NG-nitro-L-arginine –methyl ester (L-NAME); n.:10): these hearts were perfused with apelin-13 after ischemia as in group 3, were treated with 100 µM of the NO synthase (NOS) inhibitor L-NAME (Sigma-Aldrich,Switzerland) 5 min before ischemia and during the first 25 min of reperfusion bracketing the 20-min infusion of apelin.

Assessment of myocardial injury:

Infraction size was measured by the tetrazolium technique (Nitro tetrazolium blue choloride stain: Bio Basic INC, USA) ***(fig 2).*** In brief, at the end of the experiments, each heart was rapidly removed from the perfusion apparatus, and the left ventricle was cut in 1–2-mm-thick circumferential slices. Following 20 min of incubation at 37°C in 0.1% solution of tetrazolium in phosphate buffer (8.0 gm/L NaCL, 0.2 gm/L Kcl, 1.44gm/L Na_2Hpo_4 and 0.24 KH_2po_4 gm/L), normal myocardium reduces TTC to a red formazan pigment and lack of staining of acutely infracted tissue. Stained viable tissue was carefully separated from unstained necrotic tissue and then weighed. Because ischemia was global, the total left ventricle mass corresponded to the risk area. The necrotic mass was expressed as a percentage of the total left ventricular mass [13]. Following the appropriate reperfusion period, the total reperfusion solution was collected for assaying lactate dehydrogenase enzyme (LDH) (Kits for LDH level estimation; Vitro Scient, Egypt), creatine kinase –MB enzyme (CK-MB) release (Kits for CK-MB level estimation; Bio-diagnostic, Egypt). As the determination of malondialdehyde (MDA) is one of the most commonly used methods for monitoring lipid peroxidation [14], the rest of the hearts were snap-frozen and stored at -20ºC until analysis of malondialdehyde (Kits for malondialdehyde level estimation Bio Basic INC, USA) [2].

Statistical Analysis:

The data were expressed as mean ± SD for quantitative variables and statistically analyzed according to the methods described by ***Kirkwood (1989)*** [15]. The statistical analysis is done by using SPSS program (version 17) (SPSS Inc. Chicago, IL, USA). Multiple comparisons against a single control group were made by one-way analysis of variance **(ANOVA).** Subsequent post hoc analysis to determine significant differences between two groups were performed by least significant difference (LSD) test. Test was considered significant at P values < 0.05.

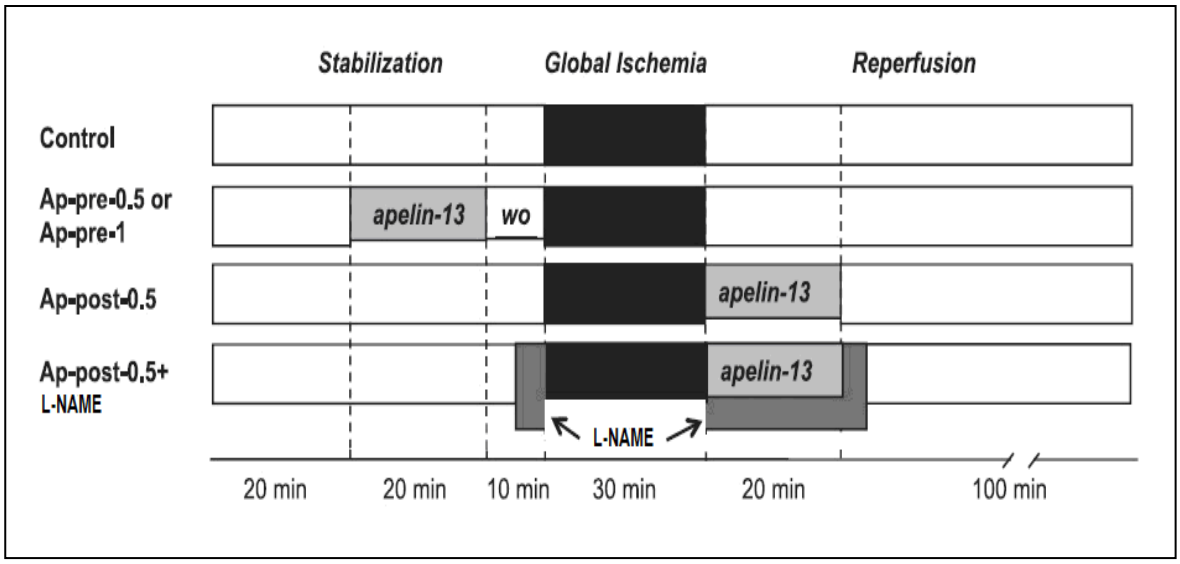

Fig. 1. Experimental protocol. The isolated Langendorff - perfused hearts after stabilization underwent 30 min of global ischemia followed by 120 min of reperfusion. Apelin-13, infusion of apelin; wo, wash out; L-NAME, infusion of NG-nitro-L-arginine –methyl ester (100 µM). Ap-pre-0.5 or Ap-pre-1, infusion of apelin-13 (0.5or 1.0 _M) for 20 min before ischemia. Ap-post-0.5, infusion of apelin-13 (0.5 _M) during the initial 20 min of reperfusion. AP-post-0.5 _ L-NNA, infusionof apelin-13 during the initial 20 min of reperfusion and infusion of L-NNA 5 min before ischemia and during the first 25 min of reperfusion.

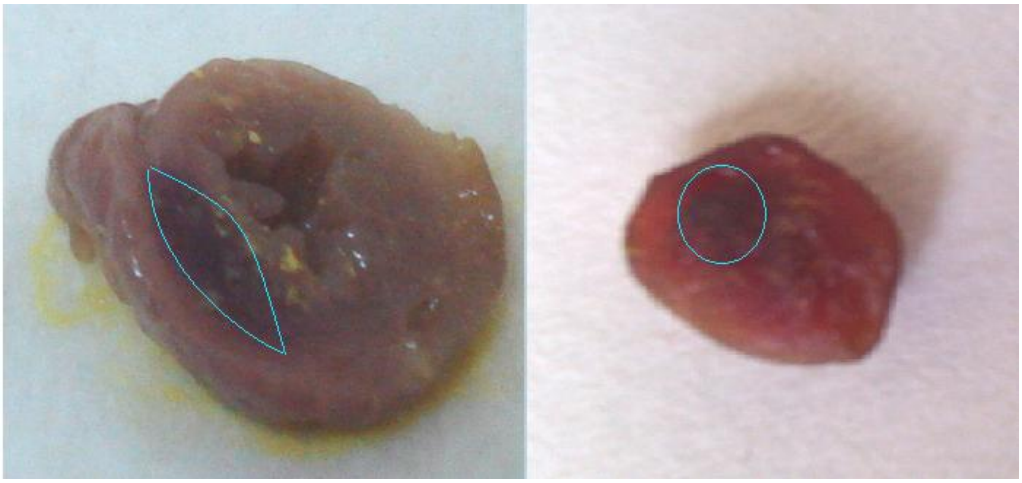

Fig. 2: Normal myocardium reduces TTC to a red formazan pigment and lack of staining of acutely infracted tissue (encircled parts in the figure).

RESULTS

Infarct size (IS):

In control hearts, IS was 56.5±4.45% of the left ventricular mass (Table 1, Fig. 3A). Pilot experiments showed that 0.1 and 0.2 µM apelin-13 during reperfusion did not affect IS (52–55% and 53–57% of the left ventricle, respectively). By contrast, IS was reduced to 22–31% and 24–33% when apelin-13 concentration was 0.5 and 1 µM, respectively. Therefore, the minimum apelin-13 concentration required to reduce IS in reperfusion is 0.5 µM. IS was not significantly changed with respect to the control when apelin-13 was given before ischemia (Ap-pre), either at 0.5 µM (53.9±4.25%) or at 1 µM (52.7±4.4%). However, IS was significantly (P <0.001) reduced to 27.6±3.72% when apelin-13 was infused after ischemia (Ap-post 0.5). In presence of L-NAME, the protective effect of Ap-post-0.5 was abolished by this NOS inhibitor (59.1±4.56%; P= ns vs. control) and IS was significantly (P <0.001) increased when compared with that produced when apelin-13 was infused after ischemia (Ap-post 0.5).

Lactate dehydrogenase enzyme (LDH) release:

In the control hearts, the cumulative LDH release collected during 2 h of reperfusion was 200±25.05 IU/L (Table 1, Fig. 3B). LDH release was not significantly different when apelin-13 was given before ischemia either at 0.5 (192.1±11.91 IU/L) or at 1 µM concentration (189±11.35 IU/L), but it decreased significantly (P <0.001) to 66.9±20.62 IU/L when apelin-13 was given during reperfusion (Ap-post-0.5). In Ap-post-0.5+L-NAME hearts, LDH release were significantly higher (233.4±38.23) with respect to control and Ap-post-0.5 hearts (P <0.01 and P <0.001 respectively).

Creatine kinase –MB enzyme (CK-MB) release:

In the control hearts, the cumulative CK-MB release collected during 2 h of reperfusion was 716.1±89.08 IU/L (Table 1, Fig. 3C). CK-MB release was not significantly different when apelin-13 was given before ischemia either at 0.5 (706.3±97.08 IU/L) or at 1 µM concentration (701.9±100.12 IU/L), but it decreased significantly (P <0.001) to 164±30.74 IU/L when apelin-13 was given during reperfusion (Ap-post-0.5). In Ap-post-0.5+L-NAME hearts, CK-MB release were not significantly different (712.5±113.98 IU/L) from control group but were significantly higher (P <0.001) with respect to Ap-post-0.5 hearts.

Malondialdehyde (MDA) release

In the control hearts, the cumulative MDA release collected during 2 h of reperfusion was 6.72±1.58 nmol/gm (Table 1, Fig. 3D). MDA release was not significantly different when apelin-13 was given before ischemia either at 0.5 (6.5±1.32 nmol/gm) or at 1 µM concentration (6.34±1.28 nmol/gm), but it decreased significantly (P <0.001) to 2.9±0.74 nmol/gm when apelin-13 was given during reperfusion (Ap-post-0.5). In Ap-post-0.5+L-NAME hearts, MDA release were significantly higher (7.98±1.17) with respect to control and Ap-post-0.5 hearts (P <0.05 and P <0.001 respectively).

Table (1): Myocardial ischemia-reperfusion injury: Infarct size, lactate dehydrogenase(LDH) release, creatine kinase –MB enzyme (CK-MB) release and malondialdehyde (MDA) release in all studied groups

	Infarction size (% of LV mass)	LDH (IU/L)	CK-MB (IU/L)	MDA (nmol/gm)
control	56.5±4.45	200±25.05	716.1±89.08	6.72±1.58
Ap- pre-0.5	53.9±4.25	192.1±11.91	706.3±97.08	6.5±1.32
Ap- pre-1	52.7±4.4	189±11.35	701.9±100.12	6.34±1.28
Ap- post-0.5	27.6±3.72***	66.9±20.62***	164±30.74***	2.9±0.74***
Ap- post 0.5 +L-NAME	59.1±4.56###	233.4±38.23**###	712.5±113.98	7.98±1.17*###

*** Significant when compared with control group (P<0.001).
** Significant when compared with control group (P<0.01).
* Significant when compared with control group (P<0.05).
Significant when compared with apelin post-0.5 group (P<0.001)

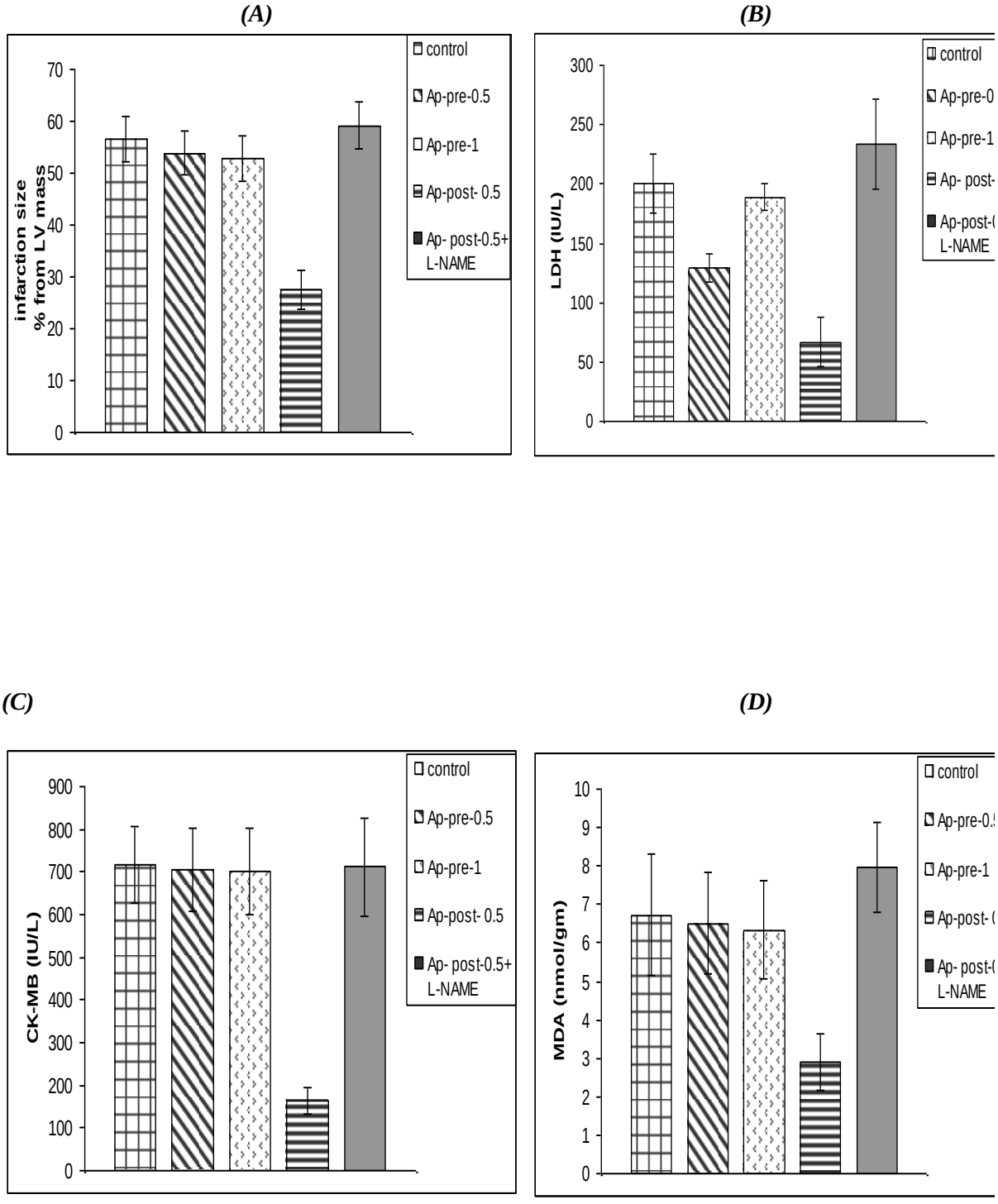

Fig. (3): Myocardial ischemia-reperfusion injury. (A): Infarct size. (B): lactate dehydrogenase(LDH) release. (C): creatine kinase –MB enzyme (CK-MB) release. (D) malonaldehyde (MDA) release

DISCUSSION

The present study showed that infraction size, lactate dehydrogenase (LDH), creatine kinase-MB (CK-MB) and malondialdhyde (MDA) levels were not significantly decreased with respect to the control when apelin-13 was given before ischemia (Ap-pre C) but, they were significantly decreased when apelin-13 was infused after ischemia (Ap-post C). Besides, it was found that infraction size was significantly decreased when apelin-13 also administrated after ischemia.

These resrults indicated that the cardioprotective effects of apelin mimic ischemic postconditioning but not preconditioning interventions as it could reduce the markers of tissue damage and decrease the infarction size when it was applied at the beginning of reperfusion.

In agreement with these findings, ***Simpkin et al. (2007)*** [(4)] found that apelin-13 when administered at reperfusion reduced infarct size both in vitro and in vivo and it delayed the opening of the mitochondrial permeability trasition pore (MPTP). They also demonstrated that apelin may be a cardioprotective agent via Reperfusion injury salvage kinase (RISK) pathway activation concerning the MPTP.

In addition, ***Zeng et al., (2009)*** [(11)] showed that apelin, through APJ receptor activation, enhanced by I/R, improves cardiac dysfunction after myocardial I/R injury by suppressing myocardial apoptosis and resisting oxidation effects. They administrated apelin to I/R hearts and found that it significantly ameliorated I/R-induced decrease in myocardial contractile function. As well, myocardial LDH leakage was markedly alleviated with apelin treatment.

The present study revealed abolishment of the cardioprotective effect of apelin in the presence of L-NAME, a nonspecific NOS inhibitor. These findings indicate involvement of NOS-dependent mechanisms in apelin-induced attenuation of myocardial I/R injury.

These findings are in agreement with ***Zeng et al., (2009)*** [(11)] who hypothesized that oxygen free radicals are a key factor involved in I/R injury. They revealed that apelin decreased reactive oxygen species (ROS) generation, malonaldialdhyde (MDA) content and increased super oxide dismutase (SOD) activity that obtained in rat heart subjected to ischemia and reperfusion and in isolated cardiomyocytes after hypoxia and reoxygenation. These findings suggest that apelin may protect the heart by alleviating oxidative injury during I/R through nitric oxide (NO).

Rastaldo et al (2011) [(12)] hypothesized that the reason whereby apelin protects the heart if given after, but not before, ischemia is due to the clear involvement of NO in the protective effect observed with apelin-postC timing and kinetics of the apelin-induced release of NO plays an important role, they also studied whether the blockade of NOS suppresses the protective effect of apelin against I/R injury. They found that NG-nitro-L-arginine (L-NNA) infusion impaired apelin-induced protection with regard to infraction size, mechanical recovery and LDH release; this finding points at NO as a mediator of the protective effects of apelin-13.

In addition ***Oleg et al., (2012)*** [(16)] demonstrated an important role of energy metabolism in post ischemic functional recovery of isolated rat heart treated with apelin. , This study revealed the reduction of the metabolic and functional response of apelin in the presence of L-NAME, a nonspecific eNOS inhibitor.

In disagreement with perivous studies ***Kleinz and Baxter (2008)*** [(10)] suggested that the RISK cascades are not always used for myocardial protection afforded by apelin and it may exhibit cardioprotection using alternative mediators and signaling pathways.

In conclusion, apelin has cardioprotective effect as post conditioning mimetic as it could reduce the markers of tissue damage and decrease the infarction size when it was applied at the beginning of reperfusion. NO is involved in this cardio protective mechanism as L.NAME could abolish the effect of apelin on I/R injury on the isolated rat heart.

Therefore, further studies are recommended on the endogenous origin and the mode of action of apelin-13 within the pool of treatments under study for postinfarction therapy as well as the use of markers for NO detection, and selective inhibitors of NOS for further delineation of apelin effects on ischemic myocardium.

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إشراك آلية أكسيد النيتريك في عمل الأبيلين علي إصابة نقص الإمداد الدموي وإعادة الإرواء لعضلة القلب في ذكور الجرذان

. إن تلف عضلة القلب الناتج عن نقص الإمداد الدموي و إعادة الإرواء يعتبر من الصور الشائعة في أمراض القلب . يمكن الحصول علي حماية ملحوظه ضد نقص الإمداد الدموي و إعادة الإرواء اما قبل نقص الامداد الدموي بفتره وجيزه او بعد فتره وجيزه من إعاده الإرواء. قد تم تسليط الضوء في الدراسات السابقة علي الأبيلين كعامل وقائي ضد تلف عضلة القلب وقد تحدث هذه الحمايه من خلال تنشيط مسارات محدده قد تعتمد اولا تعتمد علي اكسيد النيتريك. في ضوء الدراسات السابقه تم تصميم هذه الدراسة لتقييم دور الأبيلين في حمايه عضله القلب من تأثير نقص الإمداد الدموي وإعاده الإرواء وتحديد ما إذا كان أكسيد النيتريك له دور في آلية هذه الحماية.

في هذه الدراسة خضعت قلوب الجرذان المعزولة لنقص الإمداد الدموي وإعاده الإرواء الذي يتألف من 30 دقيقة من نقص الإمداد الدموي تليها 120 دقيقة من إعاده الإرواء. وقد تم تعيين هذه القلوب إلى واحد من البروتوكولات التجريبية التالية:

- **المجموعة 1 (ضابطه؛ 10جرذان):** هذه القلوب أجريت.لها عمليه نقص الإمداد الدموي و إعاده الإرواء بدون أي اضافات.
- **المجموعة 2 (10 جرذان لكل مجموعه فرعيه)**: تم تقسيم هذه القلوب الى مجموعتين فرعيتين:حيث خضعت كل منهما للإرواء مع اضافه الأبيلين 13قبل نقص الإمداد الدموي لمده 20 دقيقه ولكن بتركزين مختلفين هما 0.5و1 ميكرومول ثم تم ازاله هذا الأثر وخضعا لنقص الإمداد الدموي و إعاده الإرواء.
- **المجموعة 3: (10 جرذان):** خضعت هذه المجموعه لنقص الإمداد الدموي ثم تم إعاده الإرواء مع اضافه الأبيلين. 13 بتركيز0.5ميكرومول خلال أول 20 دقيقه من إعاده الإرواء.
- **المجموعة 4 (10 جرذان):** خضعت هذه المجموعه لنقص الإمداد الدموي ثم تم إعاده الإرواء مع اضافه الأبيلين. 13 بتركيز 0.5 ميكرومول خلال أول 20 دقيقه من إعاده الإرواء.وايضا مع إضافه 100ميكرومول من مثبطات أكسيد النيتريك 5 دقائق قبل نقص الإمداد الدموي ولمده 20 دقيقه عند إعاده الإرواء.

بعد فترة إعاده الإرواء المناسبة تم معرفه مدي التلف الناتج في عضله القلب عن طريق قياس الإحتشاء الناتج في البطين الأيسر باستخدام صبغه التترازوليم في جميع الفئات التي شملتها الدراسة. وبالإضافة إلى ذلك تم جمع محلول إعاده الإرواء في كل مجموعة لتحليل إنزيم اللاكتات نازعة الهيدروجين وإنزيم كيناز الكيرياتين . وبالإضافة إلى ذلك فإن بقية أنسجة القلب بعد قطع البطين الأيسر تم تجميدها وتخزينها عند درجه حراره -20 درجة س حتى تحليل الملونلدهيد الذي يعتبر واحدا من عوامل الإجهاد التأكسدي.

وقد أظهرت نتائج هذه الدراسة أن مستويات إنزيم اللاكتات نازعة الهيدروجين و إنزيم كيناز الكيرياتين و مستوى الملونلدهيد وكذلك حجم الإحتشاء الناتج في البطين الأيسر لم تتأثر بالمقارنه بالمجموعه الضابطه عندما أعطي الأبيلين 13 قبل نقص الإمداد الدموي بكلا الجرعتين بينما انخفضت مستويات هذه الدلالات إنخفاضا ذا دلاله إحصائيه عندما أعطي الأبيلين 13 مع بدء إعاده الإرواء. بالإضافه إلي ذلك إن كل آثار انخفاض الدلالات السابقه وأيضا حجم الاحتشاء الناتج في البطين الأيسر نتيجه إضافه الأبيلين 13 قد اختفت مع إضافه مثبطات اكسيد النيتريك.

و من مجمل هذه النتائج يتضح أن التأثيرات الواقية للقلب نتيجه الأبيلين 13 تحدث عند التدخل مع بدء إعاده الإرواء وليس قبل حدوث نقص الإمداد الدموي ويتضح ذلك من تقليل علامات تلف الأنسجة وتقليل حجم الإحتشاء في البطين الأيسر عند إضافه الأبيلين 13 مع بداية إعاده الإرواء. وبالإضافة إلى ذلك كشفت الدراسة الحالية إلغاء التأثيرات الواقية للقلب من إضافه الأبيلين 13 في وجود مثبطات أكسيد النيتريك . هذه النتائج تشير إلى تدخل آليات تعتمد علي أكسيد النيتريك في تقليل التلف الناجم نقص الإمداد الدموي و إعاده الإرواء في عضله القلب مع استخدام الأبيلين.

لذا، يوصى بمزيد من الدراسات حو ل المصادر الذاتيه للأبيلين ودوره الخاص في تقليل التلف الناتج عن إحتشاء عضله القلب و استخدام دلالات أكسيد النيتريك ومثبطات مختاره أخري لأكسيد النيتريك لمحاوله تحديد دور الأبيلين في حمايه عضله القلب.