

EXPERIMENTAL STUDY

Effects of apelin-13 on myocardial ischemia reperfusion injury in streptozotocine induced diabetic rats

Gunes I¹, Kartal H², Dursun AD³, Sungu N⁴, Polat Y⁵, Erkent FD⁶, Arslan M⁶, Kucuk A⁷

Department of Anaesthesiology and Reanimation, Erciyes University Medical Faculty, Kayseri, Turkey.
mustarslan@gmail.com

ABSTRACT

AIM: We want to investigate the protective effects of apelin-13 on myocardial ischemia reperfusion (I/R) injury. **MATERIAL AND METHODS:** 30 Wistar Albino rat were divided into 5 groups (n:6), namely control group (C), diabetes group (D), diabetes+apelin-13 group (DA), diabetes+I/R group (DIR) and diabetes I/R+apelin-13 group (DIR-A). Rats were subjected to 30-min ischemia and 90-min reperfusion. Biochemical and histopathological parameters were measured.

RESULTS: Caspase-3 enzyme activity was significantly higher in the DIR group than in the C, DA, and DIR-A groups. The intensity of caspase 3 enzyme activity was significantly higher in the I/R group than in all other groups. Inflammation and vascular dilatation were found significantly higher in the DIR group than in all other groups. Congestion was significantly higher in the DIR group than in the C and D groups. TOS enzyme activity was significantly higher in the DIR group than in the C, DA and DIR-A groups. TAS enzyme activity was significantly lower in the DIR group than in the C and DIR-A groups.

CONCLUSION: We believe that the protective effects of apelin-13 in ischemia-reperfusion injury and its use indications can be demonstrated in detail as long as the findings we have reached in our study are supported by other studies (Tab. 2, Fig. 10, Ref. 43). Text in PDF www.elis.sk.

KEYWORDS: diabetes mellitus, apelin-13, TOS, TAS, caspase-3, ischemia reperfusion.

Introduction

In the past 20 or 30 years the prevalence of diabetes mellitus (DM) has rapidly increased throughout the world, and experts estimate that it is going to increase by 200 % in the next several decades (1).

A 50 % increase in early mortality following coronary artery bypass grafting has been described in diabetic patients (2). Margolis et al showed that the rate of unrecognized myocardial infarction was 39 % in diabetic patients while in non-diabetic patients it was 22 % (3). Cardiac surgery with cardiopulmonary bypass inevitably causes a systemic inflammatory response and ischemia-reperfusion (I/R) injury affecting multiple organs (1). Several drugs have been used to prevent I/R injury, including Vitamin C, levosimendan, and dexmedetomidine (1, 4, 5).

Apelin is a recently discovered peptide encoded by APLN gene in humans and it is an endogenous ligand of the human G-protein-coupled apelin receptor (APJ); (6). The APLN gene encodes a 77-amino acid prepropeptide divided by shorter mature peptides such as apelin-13, apelin-17 and apelin-36 (7). The most studied apelins are apelin-13 and apelin-36 (8). There is increasing evidence suggesting that apelin regulates multiple physiological functions, including fluid homeostasis, food intake, cell proliferation, blood pressure regulation, angiogenesis, and glucose utilization (8, 9). Therefore, it may be associated with diabetes, obesity, hypertension and / or cardiovascular diseases (9, 10). In spite of its increased clinical use the effects of apelin-13 on myocardial injury induced by left coronary artery (LAD) I/R have not yet been investigated.

This study was carried out to investigate the possible protective role of apelin-13, which is the most active form of apelin and has the highest biological activity against oxidative damage caused by myocardial I/R in diabetic rats.

Materials and methods*Animals and experimental protocol*

This study was conducted in the GUDAM Laboratory of Gazi University with the consent of Experimental Animals Ethics Committee of Gazi University. All of the procedures were performed according to the accepted standards of the Guide for the Care and Use of Laboratory Animals.

Thirty female Wistar Albino rats (180–220 g) were used. The rats were kept at 20–21 °C in cycles of 12 hours of daylight and 12

¹Department of Anaesthesiology and Reanimation, Erciyes University Medical Faculty, Kayseri, Turkey, ²Department of Cardiovascular Surgery, Ardahan State Hospital, Ardahan, Turkey, ³Department of Physiology, Ankara University Medical Faculty, Ankara, Turkey, ⁴Department of Pathology, Yildirim Beyazit University Medical Faculty, Ankara, Turkey, ⁵Department of Cardiovascular Surgery, Mehmet Akif Ersoy Thoracic and Cardiovascular Surgery Training and Research Hospital, Istanbul, Turkey, ⁶Department of Anaesthesiology and Reanimation, Gazi University Medical Faculty, Ankara, Turkey, and ⁷Department of Physiology, Dumlupinar University Medical Faculty, Kütahya, Turkey

Address for correspondence: M. Arslan, Dr, Gazi University, Medical Faculty, Anesthesiology and Reanimation Department, 06510 Ankara, Turkey.

Phone: +90.533.4228577

hours of darkness and had free access to food until two hours before the anesthetic procedure. The animals were randomly separated into five groups, each containing six rats. Control group (C), diabetes group (D), diabetes+apelin-13 (DA), diabetes+ischemia-reperfusion (DIR), and diabetes+ischemia-reperfusion+apelin-13 (DIR-A).

Diabetes was induced by a single injection of streptozotocin (Sigma Chemical, St. Louis, MO, USA), at a dose of 55 mg/kg (i.p.) body weight. Seventy-two hours after the injection, the blood glucose levels were measured. Rats were classified as diabetic if their fasting blood glucose (FBG) levels exceeded 250 mg/dl, and only animals with FBGs of > 250 mg/dl were included in the diabetic groups (diabetes, diabetes+apelin-13, diabetes+ischemia-reperfusion and diabetes+apelin-13-ischemia-reperfusion). The rats were kept alive for four weeks after streptozotocin injection to allow chronic diabetes to develop before exposing them to I/R (11). All the rats were weighed before the study. Rats underwent left thoracotomy and received apelin-13 (i.p.; apelin-13 trifluoroacetate salt, Sigma Aldrich), 10 µg/kg, administered via i.p. route 30 minutes before ligating the LAD (13). A small plastic snare was threaded through the ligature and placed in contact with the heart. The artery could then be occluded by applying tension to the ligature (30 minutes), and reperfusion was achieved by releasing the tension (90 minutes). However, after the above procedure, the coronary artery was not occluded or reperfused in the control, diabetic and diabetic+apelin-13 rats.

Rats were anesthetized with 100 mg/kg (i.p.) ketamine. The trachea was cannulated for artificial respiration. The chest was shaved and each animal was fixed in a supine position on the operating table. The chest was opened by left thoracotomy followed by sectioning the fourth and fifth ribs about 2 mm to the left of the sternum. Positive-pressure artificial respiration was started immediately with room air, using a volume of 1.5 ml/100 g body weight at a rate of 60 strokes/min. Sodium heparin (500 IU/kg) was administered through the peripheral vein in the tail.

After the pericardium was incised, the heart was exteriorized with gentle pressure on the right side of the rib cage. An 8/0 silk suture attached to a 10-mm micropoint reverse-cutting needle was quickly placed under the left main coronary artery. The heart was then carefully replaced in the chest and the animal was allowed to recover for 20 minutes. All the rats were given ketamine 100 mg/kg (i.p.) and intracardiac blood samples were taken.

Homogenization of tissues

The heart tissue was collected into a sterile eppendorf tube and kept at -80 °C until being analyzed for total antioxidant/oxidant status. Without being allowed to dissolve, the tissues were quickly weighed on a precision scale and separated as 80–100 mg using a no. 22 lancet (PLUSMED®). Tissue fragments were smashed in the presence of liquid nitrogen in a porcelain bowl. The powdered tissue was transferred to the homogenization tube and for each gram of tissue, 140 mM KCl solution was added such that the dilution was 1/10. In order not to increase the temperature, a homogenization tube was held in a snow-filled glass beaker and after homogenization with a homogenizer (Glas_Col K5424®) at a speed of 50 revolutions per minute (rpm) for two minutes, the homogeni-

zation process was completed. The homogenate was transferred to an eppendorf tube. Eppendorf tubes were coated with parafilm and then centrifuged (Hettich Micro 200R®) for 10 minutes at 3000 rpm. After centrifugation, the supernatant was taken into another eppendorf tube and TOS and TAS levels were measured.

Total antioxidant status (TAS)

TAS was measured by a TAS test kit (RelAssay Diagnostic®, Turkey). For TAS measurement, as described in kit's procedure, 500 µL of reagent 1 (measurement buffer) and 30 µL of sample were mixed and absorbance was measured at 660 nm by a spectrophotometer (NanoDrop® ND-1000) (A₁). A volume of 75 µL of reagent 2 (colored 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid; ABTS) was added to the mixture in the Eppendorf tube. The tube was coated with paraffin and incubated for 5 minutes in a hot water bath at 37 °C (Lightning Laborteknik®). After incubation, absorbance measurement was performed at 660 nm (A₂). For standard measurement, the Trolox Eq solution at a concentration of 1 mmol / L was used instead of the sample. The first and second measurements were made for three times and their averages were measured. The absorbance change (ΔAbs) was calculated by subtracting the first absorbance value (A₁) from the second absorbance value (A₂). TAS levels were calculated using the formula given in the kit and expressed as mmol Trolox Eq / L.

$$TAS = [(\Delta Abs_{H_2O} - \Delta Abs_{Sample}) / (\Delta Abs_{H_2O} - \Delta Abs_{Standard})]$$

Total oxidative status (TOS)

TOS was measured by a TOS test kit (RelAssay Diagnostic®, Turkey). For TOS measurement, as described in kit's procedure, 500 µL of reagent 1 (measurement buffer) and 75 µL of sample were mixed and absorbance was measured at 530 nm by a spectrophotometer (NanoDrop® ND-1000; A₁). A volume of 25 mL of reagent 2 (Pro-chromogenic solution) was added to the mixture. The tube was coated with paraffin and incubated for 5 minutes in a hot water bath at 37 °C (Lightning Laborteknik®). After incubation, absorbance measurement was performed at 530 nm (A₂). A standard solution containing 10 µmol / L hydrogen peroxide (H₂O₂) equivalent / liter given in the kit was used for standard measurement. The first and second measurements were made for three times and their averages were measured. The absorbance change (ΔAbs) was calculated by subtracting the first absorbance value (A₁) from the second absorbance value (A₂). TOS levels were calculated using the formula given in the kit and expressed as mmol H₂O₂ Eq / L.

$$TOS = [(\Delta Abs_{sample}) / (\Delta Abs_{standard})] \times \text{Standard Concentration (10 } \mu\text{mol / L)}$$

Histochemical and immunohistochemical evaluation

At the end of each experiment, one of the myocardial tissue in each rat was fixed in 10% buffered formalin for 48 h, dehydrated in a graded ethanol series, cleaned in xylene, and embedded in paraffin wax, and sections of 5-µm thickness were cut using a sliding microtome (Leica Microsystems, Germany). Tissue sections were stained with hematoxylin-eosin (H&E).

Tab. 1. Histological parameters (mean ± SE).

	Group C (n=6)	Group D (n=6)	Group DA (n=6)	Group DIR (n=6)	Group DIR-A (n=6)	P**
Caspase-3 (1: less than 50%, 2: more than 50%)	1.00±0.00*	1.20±0.20	1.00±0.00*	1.60±0.25	1.00±0.00*	0.028
Caspase-3 (1: poor staining, 2: strong staining)	1.00±0.00*	1.40±0.25*	1.20±0.20*	2.00±0.00	1.20±0.20*	0.005
Edema	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	–
Inflammation	0.00±0.00*	0.20±0.20*	0.00±0.00*	0.80±0.20	0.00±0.00*	0.001
Fibrosis	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	–
Vascular dilatation	0.00±0.00*	0.60±0.40*	0.00±0.00*	1.80±0.20	0.40±0.40*	0.001
Congestion	1.00±0.00*	1.40±0.25*	1.60±0.25	2.00±0.00	1.60±0.20	0.021
Steatosis	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	–

p**: significance level with Kruskal–Wallis test $p < 0.05$, * $p < 0.05$: compared with Group DIR

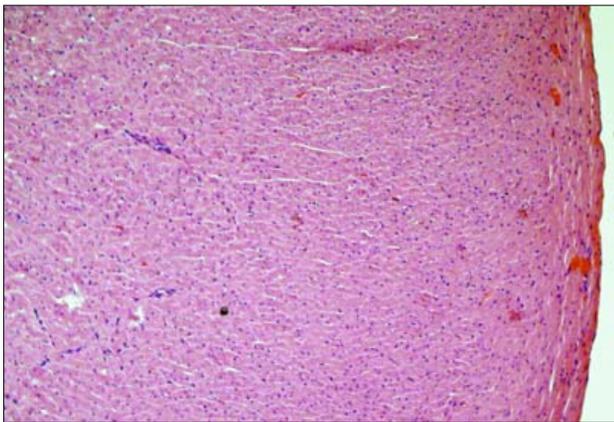


Fig. 1. Control Group. Conjugate vein structures are observed in the heart muscle. Edema, inflammation, fibrosis, vascular dilatation and fatigue are not observed in heart-muscle cells (H&E100).

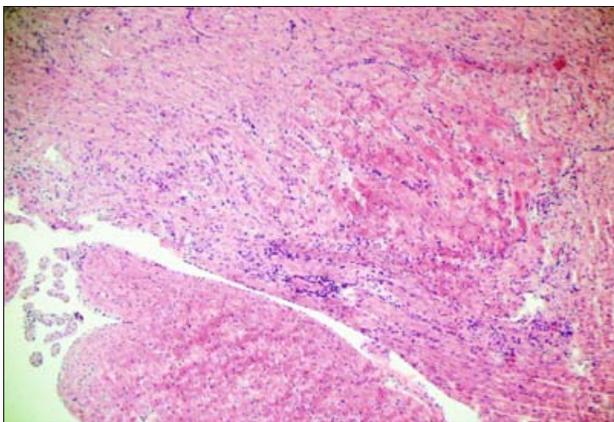


Fig. 2. Diabetes group. Severe inflammation of the interstitial area is observed in the heart muscle (H&E100).

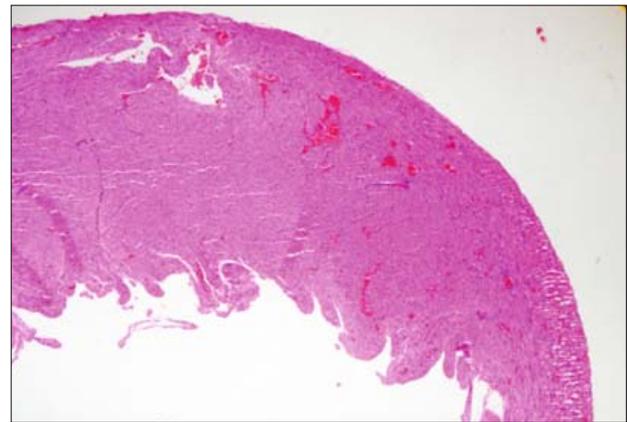


Fig. 3. Diabetes+apelin-13 group. Vessels containing mild congestion are observed in the heart muscle (H&E40).

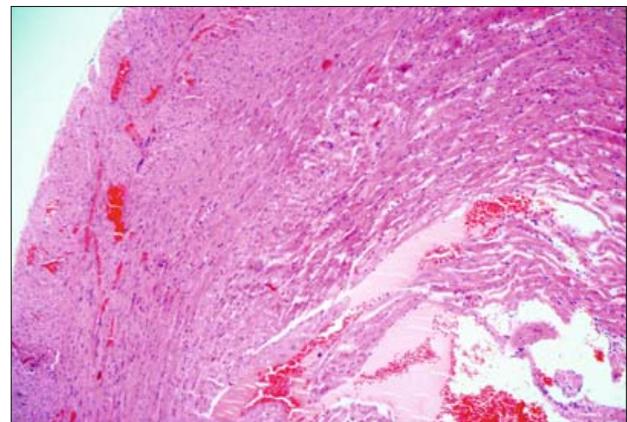


Fig. 4. Diabetes+ischemia-reperfusion group. Scoring 2 congestion and dilatation vein structures are observed in the heart muscle (H&E100).

All the sections were performed by the same pathologist who was blinded to the study. The scoring system was used for histopathological evaluation of the myocardial tissues. Interstitial edema, congestion, vascular dilatation, inflammation, fibrosis and steatosis were scored from 0 to +2 for each parameter and the total of these parameter scores was used for determining the final tissue damage. The scores of each parameter represented the severity of the pathological finding as follows: 0: no pathologic lesions, 1: low finding, 2: high pathological finding in myocardial tissue section.

Immunohistochemical evaluation

For immunohistochemical studies, complete surface sections of 3–4 μ L of poly-L-lysine-coated slides were prepared from formalin-fixed paraffin-embedded blocks of biopsy specimens in all of the events. The sections were left overnight at 45 degrees. Standard antigen retrieval methods, namely deparaffinization, blocking, primer antibody (Caspase-3: Citrate 30 min), post-primer, and polymer, DAP were used in Bond-maximal immunohistochemistry (Leica) device for Caspase-3 (p11, C-6, 1/400,

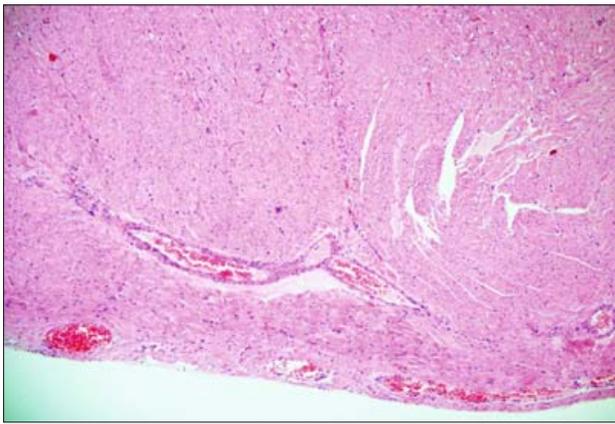


Fig. 5. Diabetes+ischemia-reperfusion+apelin-13 group. Vessels containing mild congestion are observed in the heart muscle (H&E x100).

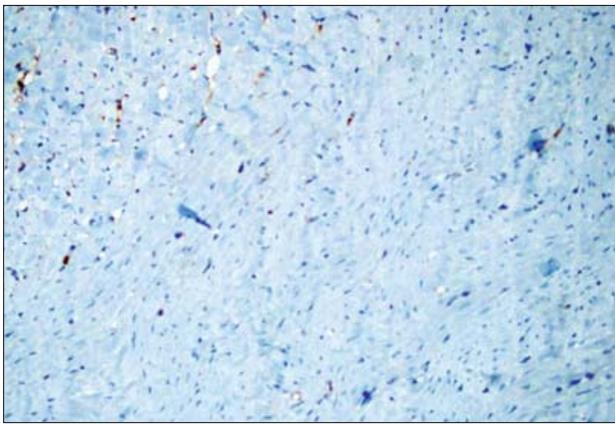


Fig. 6. Control group. Less than 50 % of vascular endothelia are weakly stained with caspase-3 (caspase-3x200).

mouse monoclonal antibody) antibodies immunohistochemically. Hematoxylin II and blue reagent were applied in the background dye for 6 and 4 min, respectively.

Immunohistochemical staining preparations were washed in water and alcohol and after being clarified with xylene they were covered with balsam. The intensity and severity of immunohistological staining in vascular endothelium were evaluated with Caspase-3. The intensity was scored as follows: 0: no staining, 1: less than 50 %, 2: more than 50 %. The severity was scored as follows: 0: no staining, 1: poor staining, 2: strong staining.

Statistical analysis

All the data were processed by variance analysis in the Statistical Package for the Social Sciences (SPSS, Chicago, IL, USA)

Tab. 2. Oxidative Status Parameters (mean \pm SE).

	Group C (n=6)	Group D (n=6)	Group DA (n=6)	Group DIR (n=6)	Group DIR-A (n=6)	P**
TOS (mmol H ₂ O ₂ , Eq/l)	6.95 \pm 1.59*	12.30 \pm 3.68	9.94 \pm 5.22*	16.57 \pm 3.04	8.45 \pm 4.64*	0.019
TAS (mmol Trolox Eq/l)	1.14 \pm 0.78*	0.63 \pm 0.09	0.63 \pm 0.12	0.36 \pm 0.09	0.93 \pm 0.46*	0.032

p**: significance level with Kruskal–Wallis test $p < 0.05$, * $p < 0.05$: compared with Group DIR

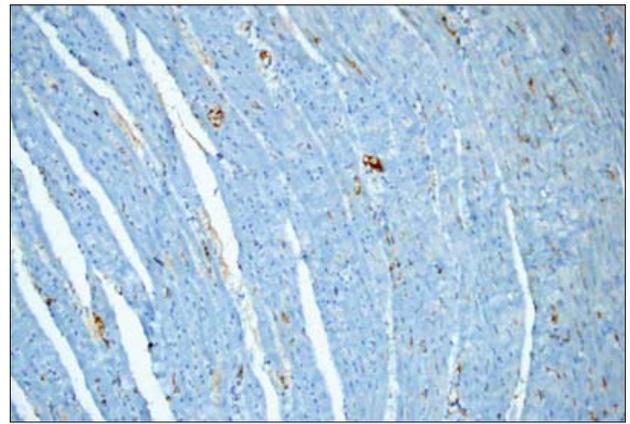


Fig. 7. Diabetes group. In the presence of severe inflammation and mild congestion, caspase-3 has weaker staining in more than 50 % of vascular endothelium (caspase-3 x200).

20.0 program for Windows statistical software. Kruskal–Wallis test was used to assess the results. Bonferroni-adjusted Mann–Whitney U test was used after significant Kruskal–Wallis to determine which group differs from the other. The data were expressed as mean \pm standard error (Mean \pm SE). Value of $p < 0.05$ was considered statistically significant.

Results

Caspase-3 enzyme activity was significantly higher in the DIR group than in the C, DA, and DIR-A groups ($p = 0.007$, all). The intensity of caspase-3 enzyme activity was significantly higher in the DIR group than in all other groups ($p < 0.0001$, $p = 0.020$, $p = 0.003$, $p = 0.003$, respectively) (Tab. 1, Figs 1–5).

Inflammation and vascular dilatation were found significantly higher in the DIR group than in the other groups. Congestion was significantly higher in the DIR group than in the C and D groups ($p = 0.001$, $p = 0.037$, respectively) (Tab. 1, Figs 6–10).

TOS enzyme activity was significantly higher in the DIR group than in the C, DA, and DIR-A groups ($p = 0.001$, $p = 0.013$, $p = 0.003$, respectively). TAS enzyme activity was significantly lower in the DIR group than in the C and DIR-A groups ($p = 0.009$, $p = 0.036$, respectively) (Tab. 2).

Discussion

Myocardial ischemia and the following reperfusion is often a fatal consequence of increased oxygen requirement and/or diminished oxygen supply to the myocardium. Thirty minutes after

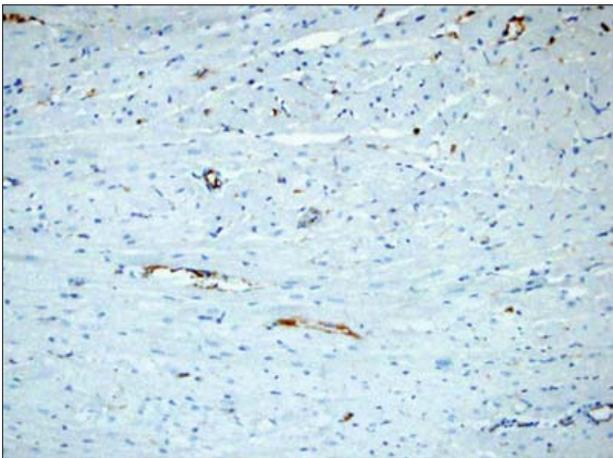


Fig. 8. Diabetes+apelin-13 group. There is strong staining with less than 50 % of vascular endothelia with caspase-3 (caspase-3 x200).

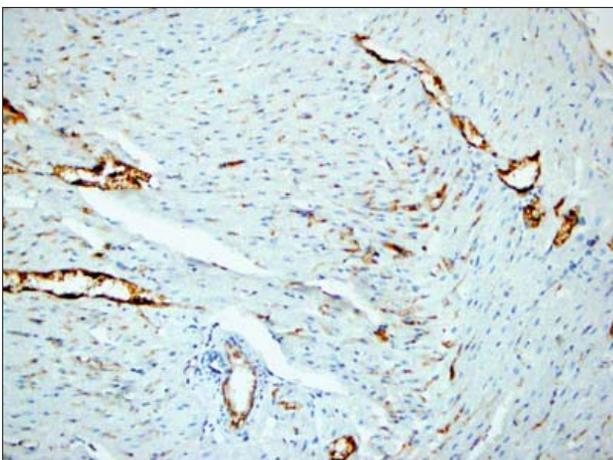


Fig. 9. Diabetes+ischemia-reperfusion group. There is more than 50 % staining of vascular endothelia with caspase-3.

coronary ligation, the signs of cellular injury start, and if duration of coronary occlusion lengthens out, the signs of irreversible myocardial injury occur, caused by contraction band necrosis and myocytolysis. After a period of ischemia (60 min) and reperfusion (60 min), edematous and nuclear changes such as chromatin clumping and swelling, apoptosis induced by increased Bcl-2, and Fas ligand are observed. Also, glycogen depletion, mitochondrial swelling, and dilatation of sarcoplasmic reticulum are observed 60 min after occlusion (12).

Yang et al (13) reported in their study that apelin could activate multiple protective mechanisms to prevent injury to the heart, brain, liver and kidney. Apelin/APJ system may be a promising therapeutic target for ischemic and other related diseases. Studies carried out report that apelin decreases the oxidative stress in the cardiomyocytes and veins of smooth muscle cells (14, 15).

Studies in mice and rats show that apelin has an analgesic role in acute pain models. (16–18). Xu et al (16) presents evidence that apelin-13 plays a significant role in the modulation of pain response at the supraspinal level in mice.

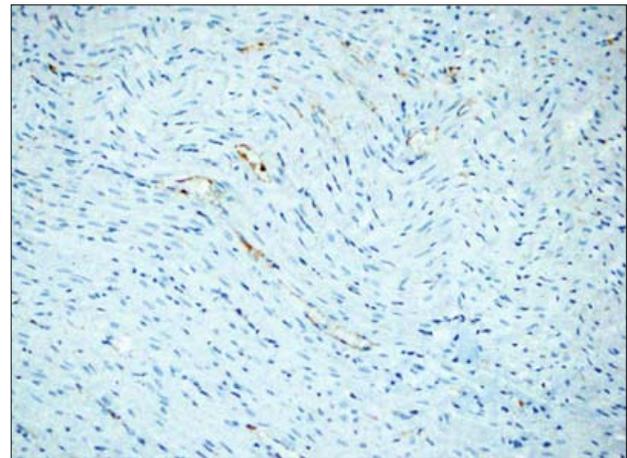


Fig. 10. Diabetes-ischemia-reperfusion-apelin-13 group. Less than 50 % of vascular endothelia are weakly stained with caspase-3.

Another study has also similar results, indicating that intracerebroventricular and intrathecal (but not intraperitoneal) injection of apelin-13 induced an antinociceptive effect in the acetic acid-induced visceral pain in a mouse model. The APJ receptor and μ -opioid receptor were involved in the antinociception of i.c.v. apelin-13 and apelin-13 (0.3 μ g) significantly potentiated the analgesic potencies of morphine (17).

Turtay et al (18) aimed herein to compare the possible analgesic effects of apelin-13 and morphine, and to reveal the mechanistic pathways underlying the analgesic effects of apelin-13 co-administered with serotonin and nitric oxide, in a rat model indexed by analgesic response times during hot-plate and tail-flick tests. Their results demonstrate that apelin-13 exerts an analgesic effect; co-administration of apelin-13 and ondansetron inhibits antinociception, an effect apparently mediated by five hydroxytryptamine-three (5-HT₃) receptors. These results should facilitate the analysis of the role of apelin-13 in acute pain and may open novel pharmacological interventions.

Unpaired electrons, free radicals, are highly reactive and readily take part in chemical reactions with virtually all cell components (lipids, proteins, complex carbohydrates and nucleic acids) in the body. These reactions occur through a chain of oxidative reactions to cause tissue injury. For most biological structures (like lipids, proteins, and nucleic acids), free radical damage is closely associated with oxidative damage, causing direct cellular injury by inducing lipid and protein peroxidation and damaging nucleic acids (19). I/R results in the generation of toxic reactive oxygen species (ROS) in the organs. Ischemia reduces the activity of cellular defense enzymes against ROS, and reperfusion or the introduction of oxygen further disturbs the delicate balance of oxidants/antioxidants (20) while intracellular calcium overload, adenosine triphosphate depletion, myocardial apoptosis, and endothelial dysfunction are all observed as a result of this (21, 22). Also, oxidative products such as ROS, reactive nitrogen species, hydrochloric acid, MDA, and lipid peroxides constitute TOS (23).

Serum concentrations of different oxidative components can be measured in the laboratory separately, but the measurement of these molecules is labor-intensive, requires much time, and complicated techniques, and is costly. TAS and TOS reflect the redox balance between oxidation and antioxidation. TAS measurement is an indicator of the activity of all antioxidants while TOS is an indicator of ROS (24, 25).

Oxidative stress is an oxidant-antioxidant imbalance status, due to oxidants which exceed the antioxidant capacity. OSI is the ratio of TOS to TAS and is an indicator of OS degree (24, 25). It may offer a more accurate comment for the evaluation of the change in oxidant-antioxidant balance. Mentese et al studies found a significant increase in OSI values at Rp 30 min. This finding suggested the development of oxidative imbalance or increase in the degree of OS in ONCABG patients (26).

In this study, TAS, as a marker of total antioxidant protection against the attack of free radicals in the organism and TOS, as a marker of total value of OS were used. Studies have shown that TAS measurement provides more valuable information than individual measurements of antioxidants (27, 28).

When we compared myocardial tissue in terms of TOS enzyme activity in our study, there was a significant difference between the groups. TOS enzyme activity was significantly higher in DIR group when compared with that of C, D and DIR-A groups. Oxidative stress, which is the primary actor of I/R injury, was found to be lower in the apelin-13-treated groups in the TOS measurement.

When comparing myocardial tissue in terms of TAS enzyme activity in our study, there was a significant difference between the groups. TAS enzyme activity was found to be significantly higher in DIR group than in C, D and DIR-A groups, which is parallel to the fact that antioxidant systems are impaired by I/R damage, while the low activity of TAS enzyme in the groups given apelin-13 indicates that the antioxidant systems are protected, in other words active. Kim et al (29) found that administration of 2.5 mg vitamin C during 5 days increased the TAS levels, and after the administration of 2.5 mg vitamin C for 5 days, TAS was found to be decreased. They thought that this decrease in TAS was related to a decrease in antioxidant stress. To the best of our knowledge, in this study, for the first time, it has been reported that I/R of the diabetic rat heart results in significant negative changes that can be observed in myocardial tissue and that the apelin signaling mediated important events in cardiovascular homeostasis. At the same time, apelin-13 has been shown to have a positive effect on myocardial contractility by promoting a potent positive inotropic effect and when administered at the beginning of heart ischemia it can provide varying degrees of protection against negative effects of variations in myocardial injury.

Recent studies suggested that apelin signaling mediated important events in cardiovascular homeostasis and has been shown to have a positive effect on myocardial contractility by promoting a potent positive inotropic effect (30, 31). Apelin is a vasodilator both in *in-vivo* (32) and *ex-vivo* models employing human arteries and veins (33). Accordingly, intravenous apelin administration in rodents reduces mean arterial pressure (34), systemic venous tone (35), and cardiac preload and afterload (30).

Rastaldo et al have found that apelin-13 could limit infarct size and improve cardiac post-ischemic mechanical recovery only if given after ischemia (36). Furthermore, Tao et al (37) reported that apelin-13 protects the heart against I/R injury through inhibition of endoplasmic reticulum-dependent apoptotic pathways.

Some reports have shown that ischemic pre- and post-conditioning could decrease cell apoptosis and myocardial injury, which could activate the PI3K/Akt and ERK1/2 signal, phosphorylate eNOS, p70S6K, GSK-3 β , GLUT4 and anti-apoptosis proteins such as Bcl-2, Bad, Bax, Caspase, as a result of which the delivery of Cyt-C is inhibited and cell apoptosis is blocked (38–43).

Apelin signaling may have an important role in the pathophysiology of diseases such as hypertension, heart failure, cardiovascular disease, type-2 diabetes, and obesity, although their effects and functions are still unclear. The physiological effects of apelin on diabetes are not fully known.

We can conclude that both types of apelin effectively decrease heart tissue injury following LAD clamping-induced I/R injury in the diabetic rat. Additionally, apelin-13 administered before induction of ischemia was observed to have protective effects on these alterations in myocardial I/R injury. Other aspects of these findings, including clinical significance and practical applications, merit further experimental and clinical investigation.

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