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The Beneficial Effects of Post-myocardial Infarction, Long Oral Treatment with M-2 in Preventing the Development of Cardiomyopathy in Rats

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Abstract

We have previously shown that the furnidipines' metabolite M-2 improved coronary flow during low-flow and regional ischemia *in vitro*. This resulted in reduced mortality and incidence, or duration, of severe arrhythmias in *in vivo* models.

The aim of this study was to establish the optimal period of oral treatment with M-2 for preventing or delaying the post-myocardial infarction (MI) cardiomyopathy development in rats.

The male Sprague-Dawley rats (n=120) were used to model the experimental MI *in vivo* and also to model physiological perfusion of the isolated rat heart. The MI was invoked by permanent left coronary artery occlusion. The surviving rats were treated with M2 (4 mg/kg daily) administered from 21st-28th, 21st-35th, 11-28th, 11-35th or from 6-35th day, post MI, for the routine estimation of morphological features of cardiomyopathy.

We summarized that the major vectors of the effects of treatment with M-2 were:

1) "Revitalisation" of the vessels and infarct scars.

2) Intensification of angiogenic events.

3) Inhibition of cardiomyopathic re-modeling of the myocardial tissue as a consequence of two mentioned above processes.

Rats treated with M-2 for the longest periods had complete protection from developing cardiomyopathy.

The early beginning and long treatment with M-2 was found the most effective for inhibiting the cardiomyopathic development. The good tolerance, long duration of action, low toxicity and relatively large therapeutic window, makes M-2, a promising candidate as a precursor for a new chemical class of cardio-protective drugs.

Keywords: Myocardial infarction; Cardiomyopathy; Furnidipines' metabolite (M-2); Morphology; Rat

Introduction

We reported previously [1] that the experimental model of Myocardial Infarction (MI) in rats, where the hemodynamic parameters of the heart are measured *ex vivo* (working heart set-up) and with a complementary histological estimation, could accomplish understanding of pathophysiological processes to optimize the cardioprotective strategy with potentially active drugs.

Prior findings suggested that evaluation for the optimal treatment time interval for the administration of cardio-protective drugs to prevent post-MI pathology was necessary. Furthermore, we found that the morphological processes in non-treated rats, correlated with hemodynamics, were slightly delayed and all the compensatory mechanisms (heart remodeling) were exhausted between 28 and 35 days after MI.

Our previous study with furnidipines' active metabolite M-2 proved its potential use in clinics as a cardio-protective drug [2,3].

In brief, Furnidipine (FUR) belongs to the 1,4-dihydropyridine group which has been called a "class of privileged structures" [4,5]. Dihydropyridines are known to protect the heart from stunning, ischemia and ventricular arrhythmias, but are at present mainly used to treat hypertension, partly due to their evident cardiac depressive action [6-15]. It is known that the effects of a parent drug might be different from that of its active metabolites; owing to differences in inherent properties such as structure, half-life, mechanism of action etc. A previously published study of ours using the rat working heart screening model [2] confirmed such a difference in effects of the pro-drug, FUR, and its two active metabolites, M-2 and M-3. In this study, both metabolites caused a marked increase in coronary flow while FUR evoked a significantly lesser change in this parameter. A distinct shift in flow from the aorta to the coronary arteries was observed with M-2 and to a lesser extent, with M-3.

M-2 exhibited the most vasodilatory effect but without markedly altering aortic pressure parameters. These observations led us to conclude that M-2 has a more beneficial influence on the heart than the parent drug, FUR. The cardiac depressive potential of M-2 was overcome by its advantageous vasodilatory effect on the coronary arteries.

In the next study we found M-2 improved coronary flow during low-flow and regional ischemia while favorably maintaining aortic

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pressure parameters and provided outstanding protection against deleterious effects of calcium overloading by significantly preventing a rise in left ventricular diastolic pressure and decrease in coronary flow [3].

Additionally, others have demonstrated that M-2, used in the same dose as in our study [16], protected isolated cardiomyocytes from hypoxia, depolarization induced intracellular calcium overloading and cellular shape changes evoked by veratridine [17-25].

Since *in vitro* results do not always correspond to *in vivo* outcomes, we studied also the influence of M-2 on hemodynamic parameters and ischemia and re-perfusion induced arrhythmias in an appropriate *in vivo* model of rats [26,27]. We found that the M-2 reduced mortality and the incidence and duration of severe arrhythmias while exhibiting differential influence on blood pressure which depended on the dose and timing of administration [3].

In conclusion, the models used in our study allowed us to test the beneficial influence of M-2 on mortality, pressure parameters and coronary flow, as well as the occurrence and duration of severe arrhythmias during re-perfusion and thus to speculate on the potential value of M-2 as a therapeutic agent for the protection of the infarcted heart.

Considering the all promising results of our previous studies [1-3,28] and prior data about the cardio-protective properties of M-2 [16], we decided to study the morphological effects, of the M-2 oral administration, for different periods after MI in rats evoked by permanent left anterior descending coronary artery occlusion.

The aim of the present work was to find whether the M-2 could prevent, or delay, post-MI cardiomyopathy in rats, establish the optimal treatment period.

Materials and Methods

Experimental animals

The experiments were conducted with male Sprague-Dawley rats (n=144) weighing 390 ± 15 g (Central Animal Farm, Medical University of Silesia, Katowice, Poland), kept on dark/light cycle (12 h light, 12 h dark), ambient temperature 21-23°C, with *ad libitum* access to food (Altromin 1220, Altromin GmbH, Lage, Germany) and water. The animals fasted overnight before the experiment.

The experiments were approved by the Local Bioethics Committee for Animal Use, Medical University of Silesia (NN-043-49/95). All animal testing was carried out in accordance with NIH regulation of animals care as described in "Guide for the Care and Use of Laboratory Animals" (released January, 1996).

Drugs and reagents used

The metabolite of furnidipine was used - M-2 [2,6-dimethyl-5 methoxy-carbonyl-4-(2'-nitrophenyl)-pyridine-3-carboxyliquide acid, MW 330.29]. This metabolite was supplied by Cermol S.A. (Geneva, Switzerland) and was primarily dissolved in diluted dimethylsulfoxide (DMSO; Sigma Deisenhofen, Germany) and later in water. This vehicle was used in the control group and the final concentration of DMSO never exceeded 0.1% v/v. Nevertheless, to exclude the influence of DMSO itself an additional control group was added. For oral administration, M-2 solutions were prepared in 0.4% aqueous dimethylsulfoxide and given in a volume of 5 mL kg^{-1}. Unless otherwise stated, all other reagents were of the highest purity and were supplied by Sigma Chemical Co. (Deisenhofen, Germany).

Experimental infarction in rats

MI was induced by permanent left anterior descending coronary artery (LAD) occlusion until 28 or 35 days, this being the same survival time period of these animals. The rats were anesthetized with pentobarbital (60 mg kg^{-1} intraperitoneally; i.p., Sigma, Deisenhofen, Germany), heparinized (500 I.U. 100 g^{-1} body weight i.p.) and the rectal temperature was maintained at approximately 38°C.

Surgical procedure were performed [29,30] with our own improvements described elsewhere [1,31-34].

In brief, the trachea was incised longitudinally and cannulated to allow artificial ventilation. The chest was opened under ventilation with room air (55-60% humidity, 23°C, stroke volume 0.8 ml/100 g of body weight; rate 54 strokes min with the positive end-respiratory pressure of 1 cm H_2O; Rodent VENTILATOR-UB 7025, Hugo Sachs Elektronik / HSE/, March, Germany) [35] by left thoracotomy at the fifth intercostal space and the fifth and fourth ribs were sectioned approximately 2 mm from the left margin of the sternum. After opening the pericardium the heart was not exteriorized and a sling (6/0 Prolene 0.7 suture attached to 3/8 circled BV-1 a 9.3 mm atraumatic, reverse cutting needle, EH 7406H, Ethicon GmbH, Norderstedt, Germany) was placed around LAD close to its origin (2 mm below). Then the ligature was passed through a plastic pad (polyethylene, 2 mm OD (0.5 ID), thickness 0.2 mm). The coronary artery was occluded by applying tension to the ligature while pressing the pad onto the heart surface. Tension was maintained by clamping a climb clip (Titan climb clip, LT-100, Ethicon GmbH, Norderstedt, Germany). Successful occlusion was immediately confirmed by ischaemia-induced alteration in ECG (ST-elevation e.g.) and observation of an arising pale ischemic zone below the climb clip. In addition, the size of transmural MI was confirmed after a working heart study, where the heart was removed and perfused 5 min with Evans blue solution and immersed in 2,3,5-triphenyltetrazolium chloride (TTC, Sigma, Poole, U.K.) for determination of the ischemic and infarcted area, respectively [36]. The area of MI in all animals (n=65) was 54 ± 6.8%. The ECG was recorded from standard limb leads using needle electrodes and recorded synchronously with the blood pressure curve on a high-speed chart recorder (Line Recorder TZ 4620, Laboratorni Pristroje, Praha, Czech) and displayed in parallel on a digital cardio monitor (CMK 405, TEMED, Zabrze, Poland). At the end of the operating procedure (approx. 15 min) tissues were sutured in layers (4-0 Deklene TM-II, 1.5, D-5427, Ethicon GmbH, and Norderstedt, Germany) excluding the pericardium (avoiding heart tamponade). The rats awoke a few hours after closing the thorax. The postoperative mortality rate of all rats was 7% (mainly caused by lethal arrhythmias and circulatory and/or respiratory insufficiency during the first day post-MI).

Experimental protocol

The surviving rats were randomly divided into 16groups. Group 1 was sham-operated (n=10). Groups 2-15 were divided into groups who received either M-2 (4 mg/kg) or 0.9% NaCl (volume 5 ml/kg) or 0.4% DMSO (volume 5 ml/kg) for one of 5 time periods (listed in days following the induced MI). Groups 2-4 received drug or placebo from the 21st to the 28th day after MI (n=14, n=8, n=6). Groups 5-7 received drug or placebo from the 11th to the 28th (n=14, n=8, n=6). Groups 8-10 received drug or placebo from the 21st to the 35th (n=14, n=8, n=6). Groups 11-13 received drug or placebo from the 11th to the 35th (n=12, n=8, n=6). Groups 14-16 received drug or placebo from the 6th to the 35th (n=10, n=8, n=6).

The periods of M-2 administration were based on hemodynamics and morphological findings reported previously [1]. In brief, these results showed nonlinear/three-phase development of hemodynamic changes in the non-treated rat hearts post-MI, whereas all the natural compensatory mechanisms (e.g. remodeling) in rats were exhausted between 28 and 35 days after MI.

The oral dose of 4 mg/kg of M-2 was chosen due to previous research [16], which found that the intravenous administration did not influence hemodynamic parameters, and demonstrated its antiarrhythmic effect and positive mortality benefits [2,3].

After administration periods (i.e. from 21st to 28th, 11th to 28th, 21st to 35th, 11th to 35th or from 6th to 35th day after MI), using the same procedures as described above, the animals were anaesthetized again and the hearts were excised for further morphological examination.

Determination of myocardial infarct size

Before morphological examination, the removed heart was perfused for 5 min., through a cannula inserted into the aorta, with 1 ml of Evans blue (2%; perfusion pressure 135 cm H_2O). It was then frozen at -20°C for 5-20 min, cut into 1.5-2.0 mm sagittal sections and immersed in a 1% solution of 2,3,5-triphenyltetrazolium chloride (TTC, Sigma, Poole, U.K.) in phosphate buffer (20 mM, pH 7.4) at 37°C for 5-15 min. The white area without Evans blue and TTC was considered as infarcted necrotic myocardium, the blue area normal myocardium and the red area (stained by TTC) ischemic myocardium. The myocardium was dissected according to the colours of myocardium and weighed separately. The percentage ratio of the weight of infarcted necrotic myocardium to that of total ischemic myocardium (infarcted necrotic myocardium and ischemic non-necrotic myocardium) was calculated and designated as the infarct size [36]. The area of MI in all surviving animals (n=134) was 54 ± 6.8%. All treatments and measurements were performed by an experimenter blind to the treatment group.

Morphologic examination

The infarct-related areas of the heart tissue were fixed in 4% of formaldehyde adjusted to pH 7.4. After routine processing through graded alcohols and xylene, the tissue was embedded in paraffin. Thin 5 µm paraffin sections of infarct-related areas of each sectioned heart were stained with hematoxylin-eozin and Masson's trichrome stains for light microscopic analysis. Histological slices (5 µm thick) were scanned with EDHISTECH slide scanner (3DHISTECH Kft. Budapest, Hungary) with 20x objective and stored. The images were comparatively evaluated under low and high magnification.

Statistics

The frequency of cardiomyopathy development after MI in rats was estimated. The chi-square-test ($\chi 2$; Yates) was used to estimate the significance between the incidences of cardiomyopathy development in all comparisons. In all cases differences were considered significant at $P<0.05$.

Results

No histological abnormalities in the hearts of the sham-operated animals were found.

At the 28th day post MI, the cardiomyopathic morphology was visible in the control (saline) hearts (n=8). Small fibrotic scars and a few inflammatory cells were present in the myocardial tissue. The smaller vessels were partly young, partly matured with walls composed of endothelial cells. Almost 40% of cases demonstrated the cardiomyopathic morphology in the control (0.4% DMSO from 11 to 28 day) hearts (n=6). The vessels were not numerous, young and mature. The major difference between these groups was: cardiomyopathic cases were more frequent in DMSO group, while the small vessels were seen in larger amounts in the first group (Figures 1A-1C, 2A and 2B).

At day 35 post MI, the cardiomyopathic morphology was more

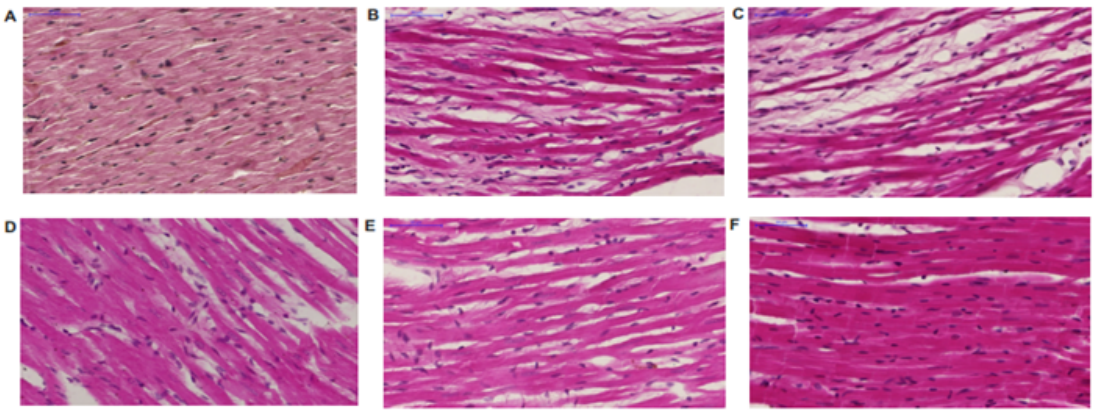

Figure 1: Representative histological findings of evaluation of myocardial tissue after infarction (MI) in rats treated with M-2 (hematoxylin and eozine – stained). (A) Control specimens of the sham-operated heart, (B) Control 0.4% DMSO water solution at 28th day after MI, (C) Control 0.4% DMSO water solution at 35th day after MI, (D) M-2 from 11 to 28 day, (E) M-2 from 21 to 35 day, (F) M-2 from 6 to 35 day (scanned with 20x objective, bar represents 50 µm).

A. Compact structure without any inflammatory infiltrations.

B. Prominent features of cardiomyopathy were seen as cardiomyocytes thinning, stretching, disperse inflammatory cells and interstitial fibrosis.

C. Progressive cardiomyopathy with fibrosis and cardiomyocytes thinning and stretching, inflammatory cells were absent.

D. Cardiomyopathic signs with focal fibrosis and disperse inflammatory cells.

E. Progressive dilation of cardiomyocytes, without progressive fibrosis and inflammation.

F. The signs of cardiomyopathy (i.e. focal fibrosis and cardiomyocytes thinning and stretching) were replaced by cardiomyocytes hypertrophy with slight inflammatory infiltration. Similar changes were visible in group receiving M-2 from 11th to 35th day after MI.

visible and distinct in the control group (n=8) in comparison to 28 days post MI. Small numbers of the vessels were present scanty and differentiated vessels were predominant. In addition, in the myocardial tissue, small scars were visible. In contrary, no cardiomyopathic morphology was present in the DMSO control group (from 11 to 35 day; n=6) in comparison to previous DMSO group (from 11 to 28 day; n=6). The inflammatory infiltrations as residual scars were composed of fibroblasts. Only new small vessels were found (Figures 1D-1F and 2C-2F).

In the group treated with M-2 from 21 to 28 days post MI (n=14), the morphology was similar only in one sample of 14 examined, while the fibroblastic scar with numerous young capillaries was seen in 9 samples. Evident signs of angiogenesis were also found. The inflammatory reaction was observed in 3 samples. In comparison to control group, the diminution of cardiomyopathic frequency and the distinct renewal of angiostructure with an angiogenesis were seen (a large number of young capillary vessels).

In the group treated with M-2 from 11 to 28 days post MI (n=14), the morphology closely resembled cardiomyopathy in 2 of 14. Visible small scars moderately vascularized and connective tissue rich in fibroblasts were seen. The vessels were predominantly differentiated, "old", in some cases inversely i.e. with the domination of young vessels. It might be concluded that evident signs of angiogenesis were present. In comparison to the control group (DMSO), the reduction of frequency of cardiomyopathic patterns was observed (Figures 1D and 2C).

In the group treated with M-2 from 21 to 35 days post MI (n=14), no cardiomyopathic features were seen and young vessels were predominantly present (>90%). Young, mainly fibroblastic, scars were observed. In comparison to control group (DMSO), the reduction of any inflammatory infiltrate was seen and the large amount of young vessels in the M-2 group was evident (Figure 1E).

In the group treated with M-2 from 11 to 35 days post MI (n=12), no cardiomyopathic features were seen and the small amount of the vessels (about 30% were young vessels) was observed. In comparison to control group (DMSO), the near complete reduction of inflammatory infiltrate was evident. The vascularization was comparable in both groups, but in M-2 group more young vessels were seen (Figure 2E).

In the group treated with M-2 from 6 to 35 days post MI (n=10), the cardiomyopathic morphology in the myocardial muscle of the ventricles was absent. Most probably all healing and differentiating processes were finished. The subepicardial granulation tissue became reduced to some old, differentiated narrow capillaries, some fibroblasts and remnants of inflammatory cells (few lymphocytes, siderophages, plasma cells). In comparison to the control group (DMSO), the domination of young vessels by young scars and the reduction of inflammatory infiltrate were characteristic and evident (Figures 1F and 2F).

The most striking and frequent pathologic lesion was the post-MI aneurysm of anterior- inferior wall of the left ventricle. These aneurysms were predominantly vascularized (Figure 3A). A peculiar finding seen in the endocardial tissues of the anesurysmal sac in two cases was the presence of chondroid tissue. It seems to be an effect of chondroid metaplasia of the endocardium or, more probably of parietal thrombus; however we did not detect any further signs of it (Figure 3B).

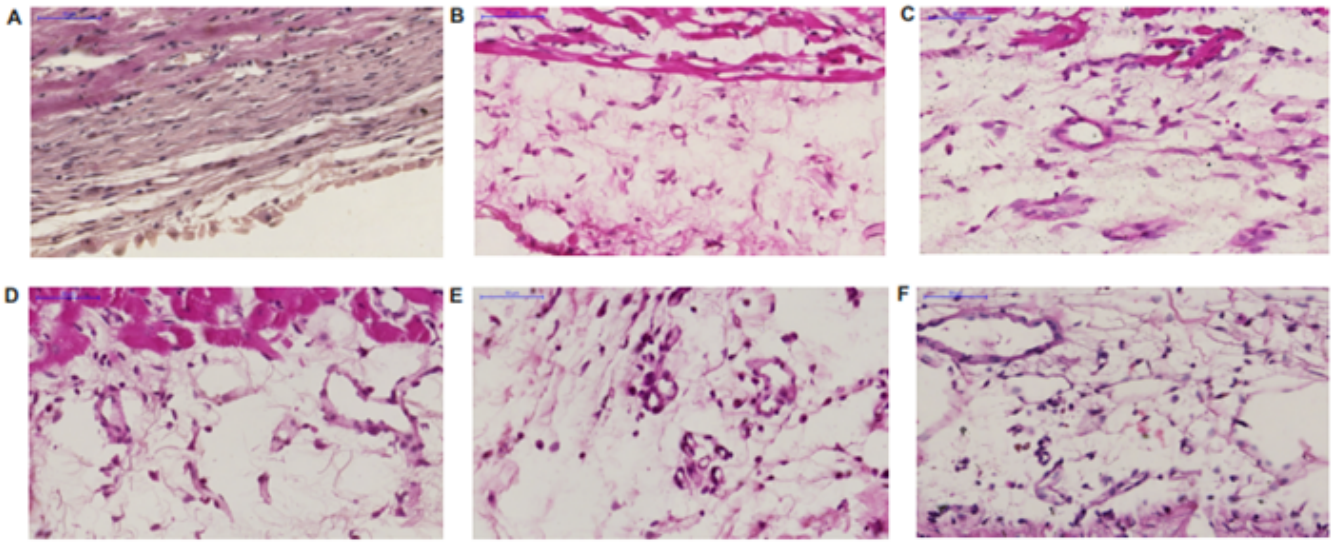

Figure 2: Representative histological findings of evaluation of epicardial vessels after infarction (MI) in rats treated with M-2 (hematoxylin and eozine – stained). (A) Control specimens of the sham-operated heart, (B) Control 0.4% DMSO water solution at 28th day after MI, (C) M-2 from 11 to 28 day, (D) M-2 from 11 to 35 day, (E) M-2 from 11 to 35 day, (F) M-2 from 6 to 35 day (scanned with 20x objective, bar represents 50 μm).

A. Left ventricular myocardium covered by fibrous tissue with single thin-walled vessels.

B. Loose fibrous tissue contains some narrow young vessels, large stimulated fibroblasts and few inflammatory cells. Similar vessels were seen after DMSO administration at 35th day after MI.

C. Thick-walled young capillary vessels were seen between fibroblasts inside young loose connective tissue.

D. Thick-walled young capillary vessels containing endothelial cells with abundant cytoplasm were characteristic inside loose epicardial tissue in this group.

E. More differentiated capillary vessels with scanty cytoplasm and young capillaries with concomitant inflammatory and mast cells.

F. Large young vessels (probably sinusoids) and numerous differentiated small capillaries and maturated fibroblasts. Few inflammatory cells.

In conclusion, it was observed that rats treated with M-2 from 21-28th day post-MI showed significant reduction of development of cardiomyopathy in comparison to the control group, which was attributed to the vehicle (DSMO). Groups treated with M-2 from the 11-28th, 11-35th or 6-35th demonstrated complete protection from development of cardiomyopathy (Table 1).

Discussion

The myocardial fibrosis phenomenon after experimental MI in rats has been previously reported, but without detailed characterization of myocardial pathology as well as the hemodynamic studies [37].

Similarly to our study, the fibrosing process (estimated by type I and III collagen mRNA) was first found on day 3 remained elevated until day 28 [37]. Cardiac hypertrophy and increase of collagen content, measured with hydroxyproline, as a result of left ventricle infarction (after coronary artery ligation) and progressive heart failure occurred in rats after 4-8 weeks in both ventricles [38].

Others authors have also reported an increase of collagen content in dogs after MI [39]. These works suggested that myocardial fibrosis is a common change after MI in the animal model, and is similar to the human response. Although the effects of permanent coronary artery ligation in rats is not quite analogous to infarction in man, the hemodynamic results and the histological evolution are similar; however, some processes are several times faster in man [1,40,41].

To better understand the reasons for such a choice of the post-MI time periods with M-2 treatment some facts should be emphasised.

In our previous study we reported [1] that at first day after MI in non-treated rats hyperemia and recent ischemic myocardial changes with few granulocytes only were seen. From day 2 the inflammatory

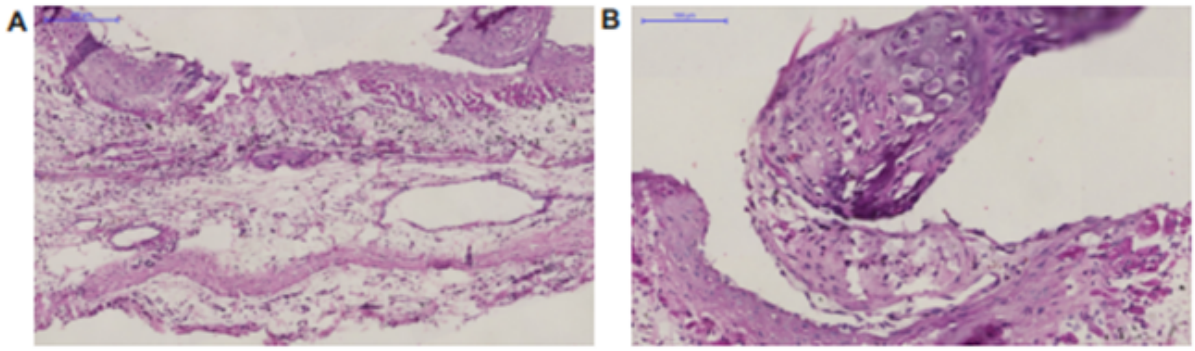

Figure 3: Additional histological findings in heart after infarction (MI) in rats treated with M-2 from 6 to 35 day (hematoxylin and eozine – stained). (A) Left ventricular aneurysm at 35th day after MI. Left ventricle in MI site is reduced to vascularized connective tissue. Small admixture of cardiomyocytes lying subendocardially (bar represents 200 μm). (B) Chondroid metaplasia in left ventricular tendinous chord. A part of chord was replaced by numerous typical chondroblasts forming a nodule (bar represents 100 μm). (Scanned with 20x objective).

Experimental groups	Time of administration [days]	Cardiomyopathy *N* [%]
Sham-operated (*n*=10)	-	0
21 to 28 day after MI		
0.9% NaCl (*n*=8)	8	8 [100%]
0.4% DMSO (*n*=6)	8	1 [16%]*
M-2 (*n*=14)	8	1 [7%]*
21 to 35 day after MI		
0.9% NaCl (*n*=8)	15	3 [38%]
0.4% DMSO (*n*=6)	15	1 [16%]
M-2 (*n*=14)	15	2 [14%]
11 to 28 day after MI		
0.9% NaCl (*n*=8)	18	8 [100%]
0.4% DMSO (*n*=6)	18	3 [50%]
M-2 (*n*=14)	18	0*#
11 to 35 day after MI		
0.9% NaCl (*n*=8)	25	3 [38%]
0.4% DMSO (*n*=6)	25	3 [50%]
M-2 (*n*=12)	25	0*#
6 to 35 day after MI		
0.9% NaCl (*n*=8)	30	3 [38%]
0.4% DMSO (*n*=6)	30	3 [50%]
M-2 (*n*=10)	30	0*#

The chi-square-test ($\chi 2$; Yates) was used to estimate the significance between the incidences of cardiomyopathy development in all comparisons. In all cases differences were considered significant at $P<0.05$; n – number of animals/hearts; *N* – hearts with cardiomyopathy.
*$P<0.05$ vs. 0.9% NaCl; #$P<0.05$ vs. 0.4% DMSO.

Table 1: Cardiomyopathy development after experimental infarction (MI) in rats treated with M-2 in the dose of 4 mg/kg p.o. daily.

infiltration was increased, but from day 6 it started to diminish and had disappeared by day 28. The necrotic cardiomyocytes were seen from day 2 to 6, and necrotic nests were replaced by granulation tissue (day 2-4), young connective tissue (day 6 to 8) and progressively collagenized scar (day 11 to 21). The regression of granulation tissue was joined with the increased number of the phagocytes (i.e. siderophages), mature fibroblasts and large, lacunar capillaries. However, a focal residual infiltration of mononuclear cells was also present. By day 11, the collagen bundles were more abundant and coarser at the areas of scar tissue than found previously. The newly formed arterioles and capillary vessels had matured and scattered inflammatory mononuclear cells were present in these areas. In addition, small clusters of mononuclear inflammatory cells were also present in the peri-infarct region as well as in the adjacent myocardium suggesting reactive myocarditis or recent-onset cardiomyopathy. At 21 days, the previously described findings were enriched by the appearance of muscle fibre disarray and hypertrophic cardiomyocytes.

The signs of myocardial response to healed MI involved cardiocytic disarray and hypertrophy, and delicate fibrosis joined with sparse inactive lymphocytic infiltrations. These changes became visible on day 21 post MI, and then became stronger suggesting cardiomyopathy on day 28. Recent cardiomyopathic findings included multifocally distributed areas of fibrosis; hypertrophy and scattered mononuclear inflammatory cells with infiltration were observed at day 28.

The cardiomyopathic morphology became more accentuated on day 35 and well established by day 70 after MI (pronounced cardiocytic hypertrophy with interstitial lymphocytic infiltration suggesting a post-MI ischemic cardiomyopathy).

Findings suggested the appropriate time intervals of M-2 oral administration to establish the optimal period of treatment for preventing or delaying the development of cardiomyopathy. The first signs of remodeling were visible on day 11, at 21 days the muscle fibre disarray and hypertrophic cardiomyocytes appeared and the recent cardiomyopathy was observed at 28 day becoming fully evident at day 35. Thus, we attempted to stop the cardiomyopathy development by varying the start of the M-2 administration (from 21th to 28th or 35th day after MI), earlier (from 11th to 28th or 35th day) and the earliest (i.e. from 6th to 35th day).

We established the relationship between hemodynamic function (working heart) and histological features of post-MI remodeling in rat heart based on combined *ex vivo* complementary studies. In our animal model the time elapsing between the non-treated infarction and the onset of cardiomyopathy may be determined hemodynamically or histologically. In brief, (1) the hemodynamic changes show nonlinear, three-phase development of the heart function post-MI in the non-treated rat ((I) up to day 4; (II) from 6 to 28 days; (III) up to 70 days), (2) the morphological processes correlate with hemodynamics, however, there are slightly delayed, (3) all the natural post-MI compensatory mechanisms (heart remodeling) in rats were exhausted between 28 and 35 days. All measured parameters remained mostly unchanged up to 70 days in comparison to day 35, while only coronary flow values raised markedly [1].

Our present histological findings could be summarized that the major vectors of the effects of treatment with M-2 were: (1) "revitalisation" of the vessels and infarct scars, (2) strong intensification of angiogenic events and (3) the breaking of cardiomyopathic re-building of the myocardial tissue as a consequence of the two mentioned above processes.

The earlier beginning and with the longest treatment with M-2 (i.e. from 6-35 day post-MI) found to be the most effective for breaking the negative remodeling sequence of the myocardial tissue.

The pirydyl compounds are able to protect the cardiomyocytes against damages induced by ionic modifications, these are responsible for most of the cardiac pathologic changes, and as such, they provided the treated animals with protection against death related to cardiac dysfunction and arrhythmias. Thus, these have potential to be cardio-protective agents in human cardiac diseases as well.

The available data to explain the molecular mechanisms of the actions of M-2 are at best suggestive. Experiments have shown that M-2 decreased the intracellular free calcium ion concentration during hypoxia in non-stimulated isolated guinea pig cardiomyocytes, which could be due to an effect on an alternative calcium entry via modified sodium channels or via sodium/calcium ions exchange [16]. M-2 also did not show a significant effect either on chronotropism and inotropism of spontaneous-beating rat atria, while it enhanced the ouabain-induced increase in systolic tension and attenuated increase in diastolic tension without affecting the normal inotropic activity in isolated rat left atria. In addition, M-2 reduced the anoxia induced shortening of action potential (>90% reduction), suggesting a possible direct action of M-2 on outward potassium ATP-dependent channels. Moreover, M-2 also reduced the veratridine-induced action potential lengthening (>90% reduction), which could possibly be attributed to the direct action of M-2 on tetrodotoxinsensitive fast sodium channels because veratridine acts by slowing the inactivation of these channels. Thus, it has been suggested that M-2 acts as a sodium and outward potassium ATP-dependent channels gating protector. In all studies, M-2 itself neither relaxed nor contracted these isolated smooth muscles as most dihydropyridines do. M-2, from the findings described above, seems to be more active at outward potassium ATP-dependent channels than at calcium channels, which could explain the lack of its cardiac depressive action [3,16,21-23].

Our present results could be explained in part only with the mechanisms described above. It needs further elucidation. For example, the most striking and frequent pathologic lesion was an aneurysm in the hearts of the longest treated group with M-2. Other targets for M-2 action are also possible.

It is well known that progressive ischemic heart failure joins with immune mediated cardiomyocyte injury, post-ischemic myocardial inflammation, seen as cellular infiltrations. In rats, 20 weeks post-MI, significantly elevated levels of gene expressions for IL-1β, IL-6 and TNFα confirmed an important role of inflammation in cardiac post-ischemic remodeling [42]. Additionally, IL-1β expression was highest and its level correlated with collagen deposition in the non-infarcted region. It is also well established that inflammation and fibrosis over the infarction site, in both ventricles, gives an assumption for histologically proved cardiomyopathy.

It seems possible that in our "cardiomyopathic" control groups (from 11 to 35 days post-MI), the promotion of angiogenesis by classical and facultative angiogens may reflect as cicatrix vascularization, stromal fibrosis and recovery of hemodynamic values leading to compensatory stabilization of the heart function after MI. Because of stimulatory effect on angiogenesis, this can be due to the M-2 activity.

This period can be considered unequivocally as the time for infarct healing, chronic ischemia or long lasting preconditioning with all consequences. The healing processes include an inflammatory reaction which was evident not only in the earlier phase of our study, but also

in the cardiomyopathic stage. These inflammatory factors can also be considered as a source of cytokines, while TNFα and interleukines participate not only in cardiomyopathy progression, but also in late phase of myocardial preconditioning [43].

The role of capillary circulation in experimental MI concerns not only its proliferation, maturation and revascularization. The elegant study [44] on capillary beds in early infarction in rats, has shown that the collateral capillary circulation became active between 15 and 30 minutes after coronary occlusion, reaching the majority of the infarcted zone at 3 h. This phenomenon is partially explained by venular back flow. The opening of collaterals may act as an additional mechanism reducing infarct size at the same time being an additional source for the neovascularization. Moreover, by rats subjected to hypoxia acting like a focal myocardial preconditioning, an increased amount of capillaries and arterioles compared with non-conditioned groups has been shown. The balance between hypoxia, myocardial injury, programmed cell death as apoptosis or autophagy, arterio- and angiogenesis is the self-stimulation. This observation is strongly supported by other works [45,46] where the amount of VEGF mRNA was higher in preconditioned groups, involving also nuclear factor κ (kappa) B-dependent pathway. Besides the "classical" angiogens like VEGF, also fibroblast growth factors are involved in the preconditioning phenomenon [47-49].

Searching for the mechanism of the cardiac remodeling after MI in rats, we have previously reported that the local expressions of TNFα, iNOS and VEGF in inflamed border zone of infarcted myocardium plays an important role in heart dysfunction and remodeling and this process is related to progressive (parallel) development of heart failure assessed by hemodynamic measurements [32,33]. In the post-MI early period a hemodynamic deterioration was most prominent and it was parallel to the expression of iNOS and TNFα mRNA (till day 11). Considering this data it has been suggested that the greatest decrease in hemodynamic function was accompanied by the expression of these two cytokines, which were continuously present till day 35 [32,33]. The suggested mechanism for participation of TNFα in myocardial insufficiency is the depression of cardiomyocytes contractility by both NO-dependent and NO-independent mechanism [50,51]. The local availability of TNFα could be attributed to the induction of iNOS and consequently high production of NO [52], which was also confirmed in our study [32,33]. In addition, the excess of NO produced by iNOS can be cytotoxic to cardiomyocytes as well as endothelium [53], however, NO could stimulate also an amelioration of the coronary bed after MI. Besides its known activities, it might be speculate that the M-2 as a potential NO donor participates in this processes.

On the other hand, the selective endothelial mitogen-VEGF was found to protect endothelial cells against TNFα induced apoptosis [45,46], while we found that VEGF mRNA was permanently expressed in the border zone of infracted myocardium [32,33]. We suggested that the rapid induction of VEGF expression caused by an increase in left ventricular end-diastolic pressure may serve to increase permeability of myocardial capillaries resulting with hyperemia and angiogenesis [32,33]. These findings confirmed the postulated involvement of studied factors in the remodeling of the myocardium and development of post-MI heart failure and correlate with present morphological data.

The presented model of one vessel coronary artery ligation leading to post-MI heart failure in rats with a subsequent morphological elaboration offers a fast and relevant experimental tool for pre-clinical study, however the simple extrapolation of obtained pharmacologic data from rat to human provides a vast pool of species-dependent reactions and effects, causing unexplained errors, in preclinical and clinical studies with potential drugs.

In conclusion, it could be said that in rats treated with M-2 from 21-28th day after MI the significant reduction of the cardiomyopathy presence in comparison to control (NaCl) (P<0.05) was most probably caused by the vehicle (DMSO), nevertheless in groups treated with M-2 from 11-28th, 11-35th or 6-35th day after MI the complete protection of cardiomyopathy development (P<0.05) was the result of M-2 itself.

In conclusion, the results of our entire studies establish a beneficial cardio-protective role of M-2 which exhibited pleiotropic effects on the ischemic or infarcted heart by imparting protection in various ways. This combined with good tolerance, long duration of action, low toxicity and relatively large therapeutic window, makes M-2, a promising candidate as a precursor for a new chemical class of cardio-protective drugs. Furthermore, its potential clinical indication should be specified.

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