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BASIC PHARMACOLOGY

Cardioprotection by Recombinant Human Erythropoietin Following Acute Experimental Myocardial Infarction: Dose Response and Therapeutic Window

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Summary. Background: **Recombinant human erythropoietin (rhEPO) protects tissue from ischemic damage, but translation of this finding into useful guidelines with respect to human trials for myocardial infarction (MI) requires a determination of the minimum effective rhEPO dose and the therapeutic window following MI.**

Method and Results: **Serial echocardiography revealed that during four weeks following MI, induced by a permanent coronary ligation in rats, the LV end-diastolic and end-systolic volumes in untreated rats expanded from 0.35 ± 0.01 and 0.14 ± 0.01 ml to 0.84 ± 0.04 and 0.61 ± 0.06 ml, respectively, and ejection fraction (EF) reduced by 50%. A single i.v. injection of rhEPO immediately following MI in a dose of 150 IU/kg was as effective as 3000 IU/kg in causing a 2-fold reduction of the number of apoptotic nuclei in the AAR 24-h later, a 2-fold reduction of the MI size measured 4 weeks later, attenuation of progressive LV dilatation and fall in EF. A 3000 IU/kg dose had similar therapeutic effects when delayed by 4, 8, or 12 h following MI, but was not effective after a 24-h delay. A single dose of 150 IU/kg was effective within 4 h post-MI, but was without effect if administered after an 8-h delay.**

Conclusion: **Cell death, final MI size, myocardial remodeling and functional decline are significantly reduced in rats by a single injection of rhEPO in a dose as low as 150 IU/kg if administered during the first 4 h after the ischemic event. Higher doses extend the therapeutic window up to 12 h.**

Key Words. **myocardial infarct, left ventricular remodeling, apoptosis, erythopoietin**

Abbreviations. **rhEPO: human recombinant erythropoietin; AAR: myocardial area at risk; MI: myocardial infarction; LV: left ventricular; EDV: end-diastolic volume; ESV: end-systolic volume; EF: ejection fraction**

Introduction

Erythropoietin (EPO), a cytokine produced by the kidney in response to hypoxia, is known to activate erythropoiesis [1,2]. Recently reported animal experiments have demonstrated that recombinant human EPO (rhEPO) was effective in protection of neural and myocardial tissues against ischemic injury [3–8]. The neuroprotective properties of rhEPO have been successfully tested in a phase II clinical trial [9]. Antiinflamatory, angiogenic and other possible mechanisms have been implicated in rhEPO induced tissue protection but only an antiapoptotic effect has been clearly demonstrated [7,8].

We have reported that a single, 3000 IU/kg i.v. injection of rhEPO immediately after permanent ligation of the left descending coronary artery in rats resulted 24 h later in a 2-fold reduction of the number of apoptotic cardiomyocytes in the area at risk (AAR). Echocardiographic follow-up during the next eight weeks revealed that rhEPO treatment prevented left ventricular remodeling and significantly attenuated the fall in ejection fraction (EF). At the end of 8 weeks of observation, histological assessment showed that myocardial infarction (MI) size in rhEPO treated rats was reduced 4–5-fold vs untreated animals [10].

A single dose of rhEPO in excess of customary therapeutic doses for anemia patients does not result in a significant elevation of red cell mass, and no adverse effects have been reported in healthy volunteers even after an exceedingly high single dose of rhEPO [11]. In our prior study, a single injection of 3000 IU/kg of rhEPO did not result in any appreciative elevation of hematocrit in rats during the following 3 weeks [10]. Nevertheless, concerns have been raised regarding the possibility of increased thrombosis due to increased production of hyperreactive platelets [12]. Therefore, prior to recommending rhEPO as a possible treatment of acute MI in clinical trials, it is

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necessary to establish the minimum effective dose of rhEPO to treat experimental MI in rats. It is also important to establish a therapeutic window for single doses of rhEPO.

We employed the same experimental model as we used previously [10], permanent coronary artery ligation in rats, and investigated the therapeutic effects of a single intravenous injection of rhEPO in doses ranging from 3000 to 50 IU/kg with a range of delays from injection immediate following coronary artery ligation to 24-h post-MI. Treated MI animals were compared with untreated and sham operated animals. The therapeutic effects were assessed by (1) measuring the number of apoptotic nuclei in the AAR 24 h after coronary artery ligation, (2) determining LV dimensions and function by serial echocardiography during four weeks following coronary ligation, and (3) assessing MI size histologically at the conclusion of the study.

Methods

Subjects, experimental groups, and experimental design

Two hundred and fourteen male Sprague–Dawley rats, 8 weeks of age (205.7 ± 1.7 g), were housed and studied in conformance with the *NIH Guide for the Care and Use of Laboratory Animals*, Manual 3040-2 (1999), with institutional Animal Care and Use Committee approval.

After baseline echocardiography, animals were randomly divided into Sham operated (S, $n = 16$) or myocardial infarction groups, in which myocardial infarction (M) was induced by a permanent coronary artery ligation. Half of the S animals ($n = 8$) and 14 rats from M groups received placebo (1 mL/kg of sterile H_2O injected intravenously immediately after operation), and were designated as "not treated" (S-NT, or M-NT). In the rest of the S ($n = 8$) and M animals, $n = 12$ for each M group (treated groups), the rhEPO (Dragon Pharmaceutical, Vancouver, Canada)* was injected immediately (<5 min) after coronary ligation, and these animals were grouped according to the dose of rhEPO administered in IU/kg (S + 3000, M + 3000, M + 1000, M + 500, M + 150, and M + 50). Two different doses, 3000 and 150 IU/kg, were also injected following delays of 4, 8, 12, and 24 h, and each of these groups (3000 or 150) were designated accordingly (M + 4, M + 8, M + 12, and M + 24). The pattern of dose/time combinations of M groups is illustrated in Figure 1. Echocardiography was repeated two more times, at one and four weeks after coronary artery ligation or sham operation, and after that all animals were killed with a bolus i.v. injection of 4 ml of 0.5 M KCl. Their hearts were harvested for histological evaluation and infarct size measurements. Groups M-NT, M + 3000, M + 150, and M + 50 included additional subsets of animals ($n = 7$ each), that were killed 24 h after coronary artery ligation to assess the apoptosis in the area at risk.

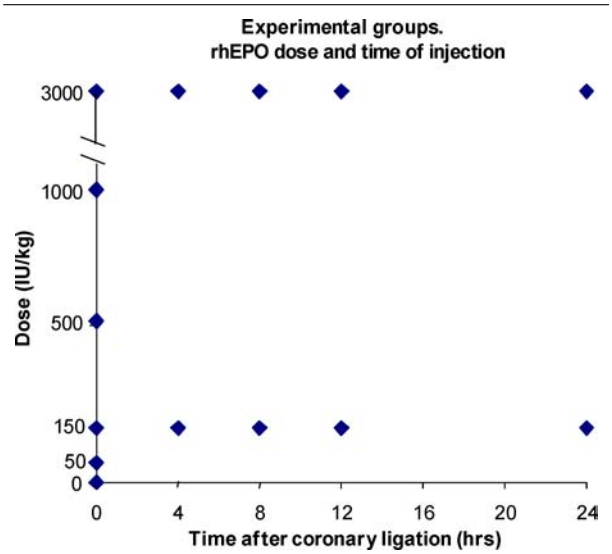

Fig. 1. *Diagram representation of experimental groups according to rhEPO dose or delay of treatment. Groups S and S-NT are not presented.*

Thus, there were 16 groups altogether, two untreated (S-NT and M-NT), one treated sham (S + 3000), and 13 MI groups treated immediately after coronary ligation with a range of different doses of rhEPO, or treated with either 3000 or 150 IU/kg injected after four different delays following coronary ligation.

Surgical procedure

General inhalation anesthesia was induced by 10% isoflurane. Rats were intubated and connected to mechanical ventilation, and anesthesia was maintained by 2% isoflurane in oxygen. Rats were subjected to ligation of the paraconal interventricular branch of the left coronary artery (corresponding to the left anterior descending coronary artery in humans) or to Sham operation, as previously described [10]. All animals received a single injection of rhEPO in the dose (IU/kg) according to their dose-designated group (see above) immediately (<5 min) after surgery, or following a delay according to their therapeutic window-designated group (see above). All doses were injected in 1 ml/kg of sterile H_2O via a femoral vein. Untreated animals received an injection of 1 ml/kg of sterile H_2O.

Doppler echocardiography

Cardiac function was assessed by echocardiography (HP Sonos 5500 equipped with a 12-Mhz phase array linear transducer, S12, which allows a 150 maximal sweep rate) under light general anesthesia with sodium pentobarbital (30 mg/kg i.p.) as described previously [10]. Parasternal long axis views were obtained and recorded to ensure that the mitral and aortic

valves and the apex were visualized. Short axis views were recorded at the mid-papillary muscle level. Endocardial area tracings using the leading edge method were performed in 2D mode (short and long axis views) from digital images captured on cineloop to calculate end-diastolic and end-systolic LV areas. LV end-diastolic volume (EDV) and LV end-systolic volume (ESV) were calculated by a modified Simpson's method from the long axis view. LV ejection fraction (EF%) was then derived as EF = (EDV – ESV)/EDV × 100. All measurements were made by a single observer who was blinded to the identity of the tracings. All measurements were averaged over three to five consecutive cardiac cycles. The reproducibility of measurements was assessed in two sets of baseline measurements in 10 randomly selected rats, and the repeated measure variability did not exceed ± 5%.

Infarct size measurement

Four weeks following MI, rats were killed by a bolus injection of 4 ml of 0.5 M KCl. Hearts were excised and placed in 10% phosphate-buffered formalin. The fixed tissue was later embedded in paraffin and serially cut from the apex to the level just below the coronary artery ligation site. Transverse 6-μm-thick sections were cut at 600-μm distances, such that 9–12 sections were obtained from each heart. Sections were stained with hematoxylin/eosin and azan, and morphometric analysis was performed by computerized video imaging using an Axioplanmicroscope (Zeiss) and NIH IMAGE software. The myocardial infarct size of each section was calculated as the ratio of infarcted area to the area of total LV section. The infarct size of all sections was averaged and expressed as the percentage MI size for each heart.

Assessment of apoptosis

Twenty-four hours after coronary artery ligation, under general anesthesia with sodium pentobarbital (50 mg/kg i.p.), 2 ml of 5% Evans blue was injected into the right ventricular chamber via the right jugular vein. Immediately after injection rats were killed by a bolus injection of 4 ml of 0.5 M KCl, and the hearts were removed, rapidly rinsed in PBS, and snap-frozen in liquid nitrogen. Serial, 6-μm-thick cryostat sections were prepared and subsequently processed for tetrazolium chloride and terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) staining. The well perfused myocardium stained by Evans blue was separated from the nonperfused part. The parts unstained by Evans blue, containing a combination of dead tissue and underperfused but viable myocardium [area at risk (AAR)], were incubated for 20 min in tetrazolium chloride then transferred in 4% paraformaldehyde. In all resulting sections, the AAR of myocardial tissue stained red, while dead tissue remained white. The AAR was further subjected to TUNEL staining for detection of apoptotic cells using a commercially available kit (Roche, Minneapolis) as directed by the manufacturer. Slides were examined by light microscopy. In each section, the number of cardiomyocytes and the number of TUNEL-positive cardiomyocyte nuclei were counted and totaled in 10 randomly selected fields of the AAR at ×400 amplification. Only nuclei that were clearly located in cardiomyocytes were counted.

Statistical analyses

Data was presented as means ± SEM. Echocardiographic changes over time were compared between groups using two-way ANOVA for repeated measurements noting the statistical significance of group × time interaction. Histological data were assessed using one-way ANOVA with a post-hoc comparison. Statistical significance was accepted at $p < 0.05$.

Results

Mortality and baseline characteristics

Total mortality among coronary artery ligated animals was 24.7%, ranging from 17 to 33% in different dose/treatment delay groups. The mortality rate, however, was not affected by the dose or time of treatment. One animal died in S-NT group. All mortalities were perioperative, i.e., occurred either during the surgery or during the first 24 h after surgery. The final number of animals in each group is noted in Table 1, which presents the average baseline values of EDV, ESV, and EF in each group measured by echocardiography. There were no statistical differences among experimental groups in either of these indices. The echocardiographic results assessing the LV remodeling and function are presented as percent changes from the baseline levels.

Dose response experiment, treatment applied immediately after coronary ligation

Echocardiography Results of dose response experiment are presented in Figure 2. Different doses of rhEPO were injected i.v. immediately (<5 min) after coronary artery ligation or sham operation. During the 4-wk observation EDV in M-NT increased 130% (Figure 2A), ESV increased 340% (Figure 2B, and EF fell 60% (Figure 2C). The rhEPO dose used in previous experiments [10], 3000 IU/kg, (M+3000) resulted in a significant attenuation of LV remodeling and functional decline – EDV increased only 90%, ESV increased 180%, and EF fell 25%. In fact, the increase of LV volume, both in end-systole and end-diastole did not differ from that of S groups, which represents the natural growth of the heart at this age. Treatment with reduced doses, including the dose as low as 150 IU/kg resulted in attenuation of LV dilation and functional decline similar to that of M + 3000. However, treatment with 50 IU/kg of rhEPO was completely

Table 1. *Baseline echo parameters of LV in different treatment groups (means ± SEM)*

	Groups (*n*)	EDV (ml^3)	*SEM*	ESV (ml^3)	*SEM*	EF (%)	*SEM*
3000 IU/kg	S-NT (7)	0.347	± *0.016*	0.138	± *0.004*	59.9	± *0.9*
	S+ (8)	0.341	± *0.015*	0.142	± *0.008*	58.5	± *1.2*
	Dose (IU/kg)						
No delay	M - NT (10)	0.355	± *0.011*	0.140	± *0.005*	60.5	± *0.8*
No delay	M+3000 (8)	0.326	± *0.011*	0.130	± *0.005*	60.0	± *1.3*
No delay	M+1000 (8)	0.330	± *0.011*	0.124	± *0.005*	62.4	± *0.8*
No delay	M+500 (9)	0.354	± *0.018*	0.138	± *0.008*	60.9	± *1.0*
No delay	M+150 (8)	0.336	± *0.009*	0.131	± *0.005*	61.1	± *0.6*
No delay	M+ 50 (9)	0.400	± *0.016*	0.158	± *0.008*	60.5	± *0.5*
	Delay (hrs)						
3000 IU/kg	4 HR (7)	0.351	± *0.010*	0.136	± *0.004*	61.2	± *0.8*
3000 IU/kg	8 HR (10)	0.360	± *0.007*	0.141	± *0.003*	60.7	± *0.8*
3000 IU/kg	12 HR (9)	0.380	± *0.015*	0.152	± *0.005*	59.8	± *0.8*
3000 IU/kg	24 HR (8)	0.362	± *0.014*	0.135	± *0.005*	62.4	± *1.2*
	Delay (hrs)						
150 IU/kg	MI+4 h (9)	0.480	± *0.006*	0.200	± *0.003*	58.3	± *0.2*
150 IU/kg	MI+8 h (10)	0.420	± *0.013*	0.166	± *0.006*	60.6	± *0.3*
150 IU/kg	MI+12 h (9)	0.387	± *0.007*	0.155	± *0.003*	59.9	± *0.2*
150 IU/kg	MI+24 h (10)	0.474	± *0.011*	0.197	± *0.006*	58.6	± *0.5*

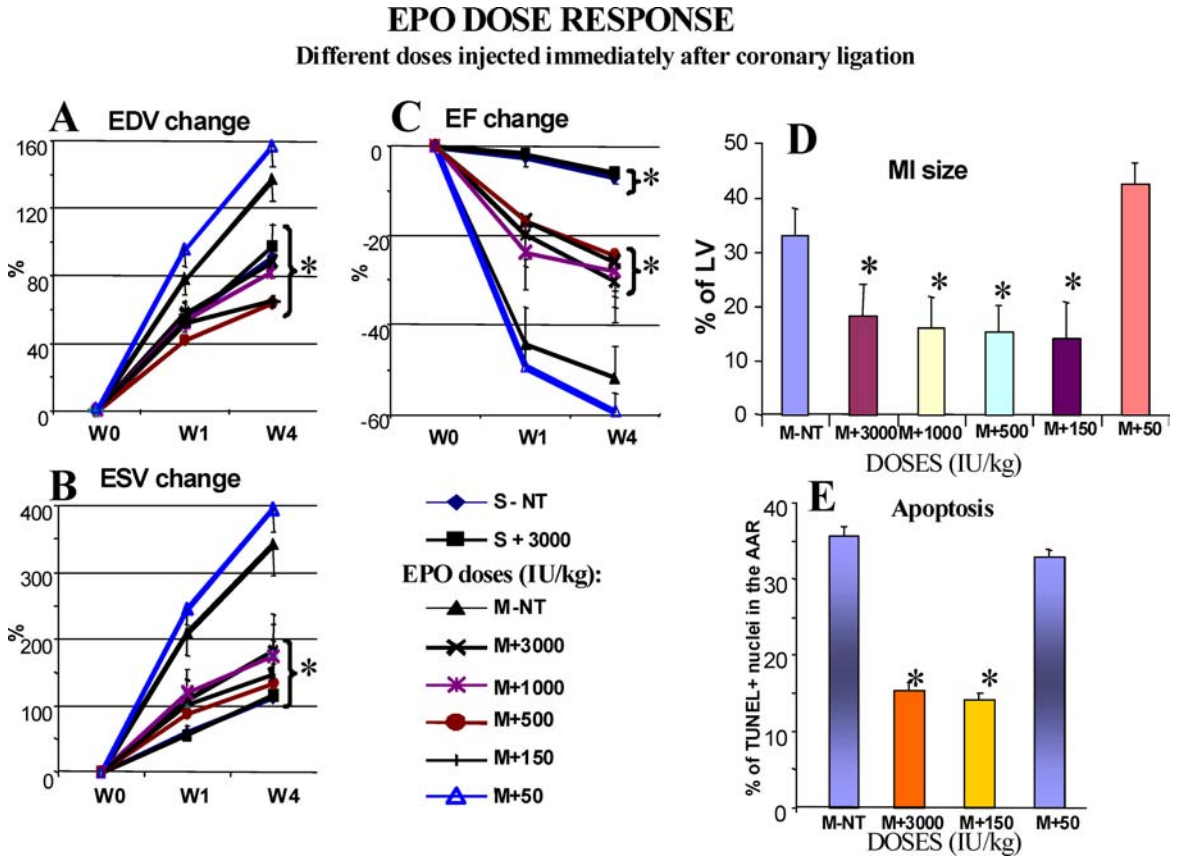

Fig. 2. *Response to rhEPO injection immediately after coronary ligation in doses 3000, 1000, 500, 150, and 50 IU/kg. Average echocardiographic measurements of EDV (A), ESV (B), and EF (C) changes during four weeks following permanent coronary artery ligation, histologically measured MI sizes at the end of four weeks (D), and percent of apoptotic cardiomyocytes in the AAR 24 h after coronary ligation (E). * significantly different from M-NT ($p < 0.05$): ANOVA, time × group interaction (A–C); ANOVA, post hoc comparisons (D, E).*

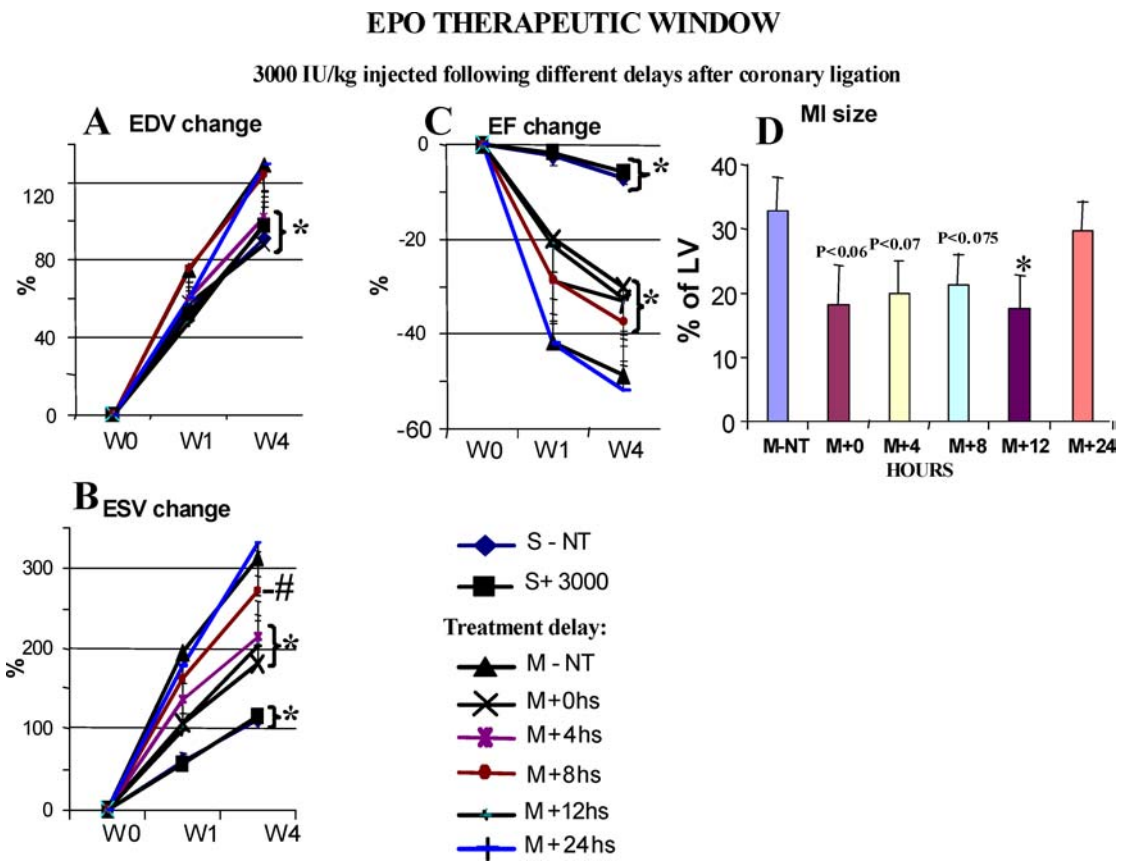

Fig. 3. *Response to 3000 IU/kg of rhEPO injection immediately after coronary ligation and after 4, 8, 12, and 24 h of delays. # ANOVA time × group interaction, $p < 0.07$ (B). In the graph representing histologically measured MI sizes (D) the overall ANOVA $p < 0.05$. The rest is the same as in Figure 2.*

ineffective—the results for M + 50 were not different from that of M-NT.

MI size Histologically measured MI sizes in each of the dose groups at the end of 4-wk post MI observation are presented in Figure 2D. The size of MI in the M + 3000 is approximately half of that in M-NT. The MIs in other dose groups are equally reduced and similar to that in M + 3000, with the exception of M + 50, in which the MI is as large as in untreated group.

Apoptosis in the AAR 24 h after coronary ligation

The number of apoptotic nuclei 24 h after coronary ligation was determined in the AAR in subgroups of rats from four groups, M-NT, M + 3000, M + 150, and M + 50 (Figure 2E). The percent of apoptotic nuclei in experimental groups treated with the highest and lowest effective doses (3000 and 150 IU/kg), as demonstrated by the reduction of LV remodeling, functional decline, and MI size four weeks following coronary artery ligation, was similar and represents about the half of apoptotic nuclei in the AAR of untreated animals (M-NT). The apoptosis in M + 50 was equal to that in M-NT, i.e., the dose which was ineffective with respect to remodeling and MI size, was equally ineffective in reduction of apoptosis in the AAR 24 h after coronary ligation.

Therapeutic window experiment at the highest effective dose (3000 IU/kg)

Echocardiography Figure 3 represents the results of the therapeutic window experiment. The highest effective single dose of rhEPO demonstrated in dose response experiment, 3000 IU/kg, was injected with different delays following artery coronary ligation. The EDV, ESV, and EF changes in M-NT, M + 3000, and two S (treated and untreated) groups as described above are reproduced here from Figure 2 for comparison. Note that the group denoted as M + 3000 in Figure 2, is denoted here as M + 0. The treatments delayed by 4, 8, and 12 h (groups M + 4, M + 8, and M + 12) were as effective with respect to at-

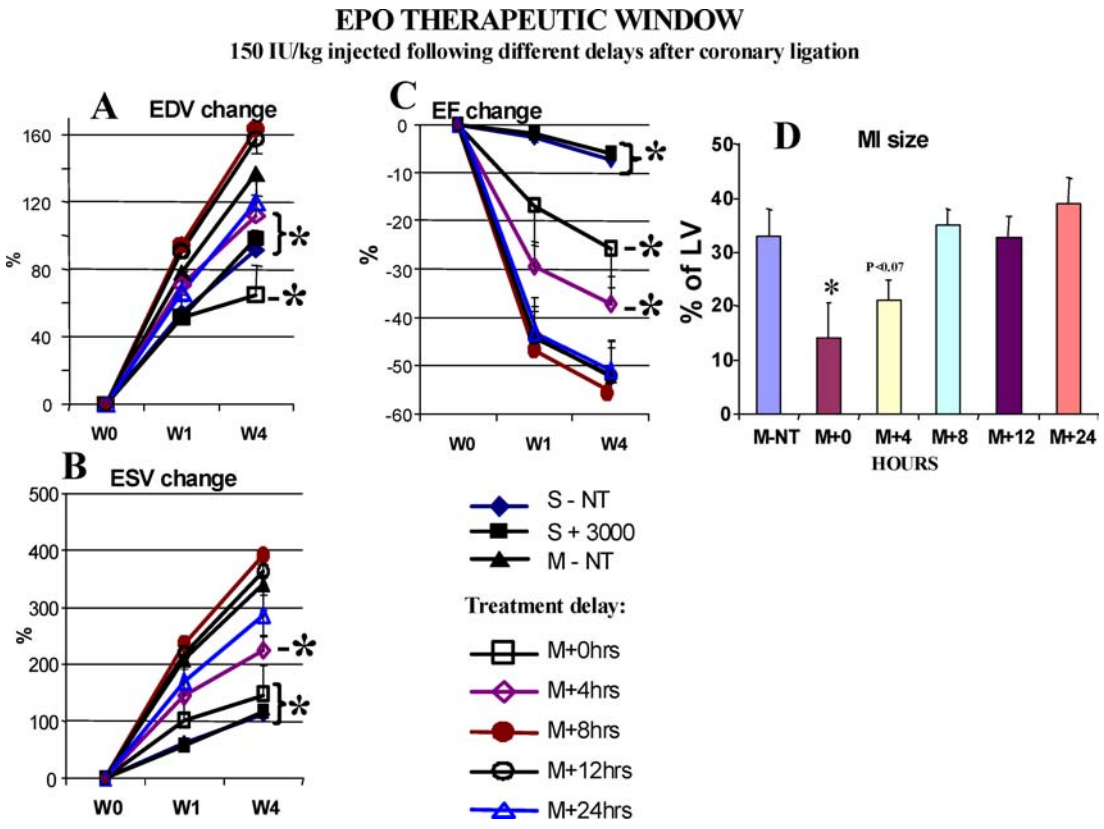

Fig. 4. *Response to 150 IU/kg of rhEPO injection immediately after coronary ligation and after 4, 8, 12, and 24 h of delays. The rest is the same as in Figure 2.*

tenuation of LV dilation and EF fall as treatment applied immediately after surgery (M + 0). Changes in their sonographic indices were all statistically different from M-NT, but did not differ from each other. M-8 was the exception, in which changes in EDV were not different from M-NT, and changes in ESV did not quite reach a statistically significant difference from M-NT ($p < 0.07$). However, the fall of EF in this group was similar to that of M + 0, M + 4, and M + 12 groups. In contrast, treatment following 24 h delay (M + 24) was ineffective—the changes in EDV, ESV, and EF in this group were similar to those of M-NT.

MI size Histologically measured MI sizes at the end of 4-wk post MI observation in each of the treatment delay groups, which received 3000 IU/kg of rhEPO, are presented in Figure 3D. The MIs in M + 4, M + 8, and M + 12 are similar in size to those in M + 0 (group treated immediately after coronary ligation), and about half of that in M-NT. An exception is the group in which treatment was delayed by 24 h (M + 24). The MI size in this group was not different from M-NT. Reduction of MI size for M + 0, M + 4, and M + 8 did not reach statistical significance, but a trend was apparent.

Therapeutic window experiment at the lowest effective dose (150 IU/kg)

Echocardiography Figure 4 represents the results of therapeutic window experiment, in which the lowest effective single dose of rhEPO demonstrated in dose response experiment, 150 IU/kg, was injected with different delays following coronary ligation. The EDV, ESV, and EF changes for M-NT, M + 150, and two S (treated and untreated) groups were described above and reproduced here from Figure 2 for comparison. Note, that the group denoted as M + 150 in Figure 2, is denoted here as M + 0. The treatment delayed by 4 h (M + 4) was as effective with respect to attenuation of changes in all presented echo indices as treatment applied immediately after coronary artery ligation (M + 0), i.e., changes over time of EDV, ESV, and EF in M + 0 and M + 4 were significantly different from the changes of M-NT, but did not differ from each other. All other groups with delayed treatment at this dose (8, 12, and 24 h) showed no improvements in comparison with M-NT.

MI size Histologically measured MI sizes in each of the treatment delay groups at the end of 4-wk post MI observation are presented in Figure 4D. MI size in M

+ 4 is similar to that in M + 0 and different from M-NT ($p < 0.07$). The MI size in all other treatment delayed groups (8, 12, and 24 h) did not differ from that in the untreated group.

Discussion

Neuro-protective effects of rhEPO in the dose of 5000 IU/kg and its cardioprotective effects at a dose of 3000 IU/kg have been shown previously [3–8,10]. The therapeutic window, and lower tissue protective doses of rhEPO, have been reported in other experimental animal models. In rat models of brain damage induced by ischemia or trauma, 5000 IU/kg of rhEPO was effective even when treatment was delayed by 3 h [13], and in a rat model of kidney ischemia-reperfusion injury a dose of 300 IU/kg of rhEPO was effective [14]. The present study is the first, however, in which the interaction of both different doses and therapeutic window was evaluated in the model of permanent myocardial ischemia.

We have reported previously that a single systemic injection of 3000 IU/kg of rhEPO immediately after coronary ligation in rats significantly reduces apoptosis in the AAR of myocardium when measured 24 h later. Resulting MI size, LV dilation, and functional decline are also reduced over eight weeks of subsequent observation [10]. In the present study, conducted in the course of further preclinical evaluation using the experimental model of permanent coronary artery ligation, we established that a single injection of 3000 IU/kg immediately after ligation was as effective in attenuating LV remodeling and MI size as a single injection of the same dose delayed for up to 12 h, but was not effective at all if treatment was delayed for 24 h. In other words, the therapeutic window for this high dose of rhEPO is more that 12, but less than 24 h. If injected immediately after coronary ligation, the highest dose of 3000 IU/kg of rhEPO was equally effective in reduction of apoptosis in the AAR, LV remodeling, functional decline and resulting MI size as the lowest effective dose of 150 IU/kg. However, the therapeutic window for this low dose was significantly shortened and limited to four hours following coronary artery ligation. While the LD_{50} for rhEPO has never been reported, and no toxic dose for a single injection has been published in the Physicians' Desk Reference (2004), the presented results provide a guideline for the effective dose of rhEPO for cardioprotection relative to those used for the treatment of anemia.

Several known non-erythropoietic properties of rhEPO could contribute to a tissue protection. They are antiapoptotic, antiinflamatory, and angiogenic effects, as well as activation of progenitor cells [13,15–17]. However, with respect to cardioprotection, only the antiapoptotic effect has been documented to date [7,8] and confirmed in the present study as a putative mechanism of tissue protection by rhEPO. Indeed, among the rhEPO treated rats, the number of apoptotic cardiomyocytes in the AAR was reduced by 50% 24 h after coronary artery ligation, and proportionally, the resulting MI size four weeks later was also about 50% of that in untreated animals. The normal time course of programable death of the cardiomyocytes in the infarcted myocardium is not yet precisely established [18]. However, in experiments on adult cardiomyocytes in culture, the measurable reduction in mitochondrial transmembrane potential and initial activation of capsase-3 was detected in 2 h after stimulation with H_2O_2, and positive annexin V reaction was seen in 4 h. The first signs of DNA fragmentation appeared in 9 h. Apoptotic nuclei, characterized by chromatin condensation, could be identified in 14 h [19]. In *in vivo* experiments on rats, apoptotic cardiomyocytes were found in infarcted area starting from 3 h after coronary artery ligation and persisted at least until 36 h. No apoptotic cardiomyocytes were found in the MI area three days after coronary ligation [20,21]. Thus, the initial programmable death of the cardiomyocytes is obviously completed sometime after 36 h following the induction of ischemia. This explains the therapeutic window established in the present study: sometime between 12 and 24 h after coronary ligation the programmable cell death becomes irreversible, and too many cardiomyocytes are lost so that therapy cannot significantly affect the outcome.

There is no complete agreement yet on which specific antiapoptotic pathways are involved in cardioprotection Induced by rhEPO. Many possible antiapoptotic signaling pathways have been reported [13,22–25]. Previous findings from our laboratory showed that the PI3-kinase inhibitor, wortmannin, completely blocked the protective effect of rhEPO on mitochondrial membrane permeability threshold [26]. That finding suggests that the PI3-kinase signaling pathway is definitely involved in rhEPO-mediated protection against myocardial ischemia. In our present experiment the lowest effective rhEPO dose, 150 IU/kg, established in experiments in which rhEPO was injected immediately after coronary artery ligation, was effective if delayed by 4 h, but lost its effectiveness if delayed by eight hours, while higher dose was still effective. These results indicate that during the first four hours after coronary artery ligation the antiapoptotic pathway involved in cardioprotection is upregulated.

In summary, in the rat model of permanent coronary ligation, a single systemic injection of 150 IU/kg of rhEPO attenuates resulting MI size, LV remodeling and functional decline if applied during the first four hours after coronary ligation. By increasing the dose of rhEPO up to 3000 IU/kg the therapeutic window can be expanded for up to at least 12 h.

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