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Sodium Nitrite and Cardioprotective Effect in Pig Regional Myocardial Ischemia–Reperfusion Injury Model

Azotyn sodu i jego działanie kardioprotekcyjne na miejscowe niedokrwienie–reperfuzję mięśnia sercowego na modelu świńskim

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A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article; **G** – other

Abstract

Objectives. The present study was designed to investigate the cardioprotective effect of sodium nitrite ($NaNO_2$) and sodium nitrate ($NaNO_3$) against myocardial ischemia–reperfusion (I/R) injury in a pig regional ischemia model.
Material and Methods. Eighteen pigs were randomly divided into three groups as control (Group 1), sodium nitrite (Group 2) and sodium nitrate (Group 3) groups. Before the exploration of the heart, blood samplings were taken for alanine aminotranspherase (ALT), aspartate aminotranspherase (AST), lactate dehydrogenase (LDH), creatinine kinase–muscle band (CK-MB), troponin-t, glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT), malondialdehyde (MDA) and nitrite/nitrate (NO_2^-/NO_3^-) in all groups (T0). Following sternotomy and stabilization, blood and tissue samples were repeated (T1). Then, intracoronary sodium nitrite and sodium nitrate were given (0.5 μg/kg) in Groups 2 and 3. Five minutes later, the left anterior descending (LAD) artery was ligated for I/R experiments. Blood and tissue samplings were repeated after 60 minutes of ischemia (T2) and 180 minutes of reperfusion period (T3). Light and electron microscopic investigations were performed.
Results. There were statistically significant results in favor of Group 2 in all studied parameters. Hemodynamic parameters showed a decrease in mean arterial pressure (MAP), cardiac output (CO), cardiac index (CI) and an increase in heart rate, mean pulmonary artery pressure (MPAP), left ventricle end-diastolic pressure (LVEDP), pulmonary capillary wedge pressure (PCWP) during ischemia. Following the ischemia these parameters returned to their near normal levels. This was prominent in group 2. Oxidative parameters showed protective increases in GPx, SOD, CAT and NO_2^-/NO_3^- levels and a decrease at MDA both in tissue and blood samples in group 2. There were statistical differences only in T3 for AST, troponin-t and CK-MB levels in favor of Group 2. Histological and electron microscopy examinations were also in favor of NO_2^- group.
Conclusions. The results of the present study indicate that $NaNO_2$ provides protection against myocardial I/R injury when compared to control and $NaNO_3$ groups (**Adv Clin Exp Med 2012, 21, 6, 713–726**).

Key words: sodium nitrite, myocardium, ischemia-reperfusion injury.

Streszczenie

Cel pracy. Zbadanie działania kardioprotekcyjnego azotynu sodu ($NaNO_2$) i azotanu sodu ($NaNO_3$) na niedokrwienie–reperfuzję mięśnia sercowego na modelu świńskim.
Materiał i metody. 18 świń losowo podzielono na 3 grupy: kontrolną (grupa 1), azotyn sodu (grupa 2) i azotan sodu (grupa 3). Przed otwarciem serca pobrano próbki krwi do oznaczenia aminotransferazy alaninowej (ALT), aminotransferazy asparaginianowej (AST), dehydrogenazy mleczanowej (LDH), izoenzymu MB kinazy kreatyniny

(CK-MB), troponiny-T, peroksydazy glutationowej (GPx), dysmutazy ponadtlenkowej (SOD), katalazy (CAT), aldehydu malonowego (MDA) i azotynów/azotanów (NO_2^-/NO_3^-) we wszystkich grupach (T0). Po sternotomii i stabilizacji ponownie pobrano próbki krwi i tkanek (T1). Następnie podano azotyn sodu i azotan sodu śródwieńcowo (0,5 μg/kg) w grupach 2 i 3. Pięć minut później sklejono tętnicę zstępującą przednią (LAD), aby przeprowadzić eksperyment I/R. Pobieranie próbek krwi i tkanek powtórzono po 60 min. w fazie niedokrwienia (T2) i po 180 min w fazie reperfuzji (T3). Wykonano badanie z użyciem mikroskopii optycznej i elektronowej.
Wyniki. Nie było istotnych statystycznie różnic na korzyść grupy 2 wszystkich badanych parametrów. Parametry hemodynamiczne wykazały zmniejszenie średniego ciśnienia tętniczego (MAP), rzutu serca (CO), wskaźnika sercowego (CI) i zwiększenie częstości akcji serca, średniego ciśnienia w tętnicy płucnej (MPAP), lewokomorowego ciśnienia końcowo-rozkurczowego (LVEDP), ciśnienia zaklinowania (PCWP) podczas niedokrwienia. Po niedokrwieniu te wskaźniki wróciły prawie do normy. Było to widoczne w grupie 2. Wskaźniki oksydacyjne wykazały ochronne zwiększenie stężenia GPx, SOD, CAT i NO_{2-}/NO_{3-} i zmniejszenie MDA zarówno w tkance, jak i krwi w grupie 2. Nie było różnic statystycznych tylko w T3 dla stężenia AST, troponiny-T i CK-MB na korzyść grupy 2. Badania histologiczne i mikroskopia elektronowa były również korzystne dla grupy NO_2^-.
Wnioski. Wyniki badania wskazują, że $NaNO_2$ chroni przed uszkodzeniem I/R mięśnia sercowego w porównaniu z grupą kontrolną i grupą $NaNO_3$ (**Adv Clin Exp Med 2012, 21, 6, 713–726**).

Słowa kluczowe: azotyn sodu, mięsień serca, niedokrwienie–reperfuzja.

Administration of nitric oxide (NO) or NO donors prior to ischemia attenuates the negative consequences of myocardial ischemia/reperfusion (I/R). [1] As Gladwin and Desfluian pointed out "Nitrite (NO_2^-), historically considered inert, functions as a reservoir for NO. During physiological hypoxia and pathological ischemia, nitrite is reduced to NO, regulating hypoxic vasodilation, cellular respiration, mitochondrial reactive oxygen species (ROS) generation, angiogenesis, and cellular death programs. Nitrite in human plasma exists at concentrations of 100 to 300 nmol/L and may be reduced to NO by iron-containing enzymes, including hemoglobin, myoglobin, neuroglobin, xanthine oxidoreductase, endothelial NO synthase, mitochondrial electron transport chain proteins, and the hepatic cytochrome P450 system. The rate and extent of nitrite reduction are coupled to deoxygenation and proton generation. Thus, NO generation is coupled to oxygen and pH gradients and maximized in ischemic tissues" [2, 3].

Gladwin and Desfluian also wrote: "Nitrite therapy limits cellular injury and apoptosis after ischemia and reperfusion. Nitrite therapy is cytoprotective in numerous animal models of focal ischemia/reperfusion injury, including rodent heart, brain, liver, and kidney, canine heart, and primate brain. Systemic nitrite reduction by ceruloplasmin knockout or dietary nitrate/nitrite elimination increased infarction volume in the liver and heart after experimental ischemia. These studies indicate that physiological systemic nitrite levels modulate host resilience to ischemia" [2, 3].

In the present study, the authors aimed to investigate the cardioprotective effect of $NaNO_2$ in a pig regional myocardial I/R model and compared with the effects of $NaN0_3$.

Material and Methods

The experiment was performed in compliance with the "Principles of Laboratory Animal Care" formulated by the National Institutes of Health (National Institutes of Health publication no. 85-23, revised 1996). The experiment protocol was approved by the Ethics Committee for Animal Care, established in authors' institute (006/AR20).

Open-Chest Pig Experiment Protocol

Porcine of male gender (n = 18), mean weights 62 ± 16 kg (ranging from 49–83) were randomly divided into three groups. The first group was the control group (Group 1), the second group was $NaNO_2$ group (Group 2) and the third group was $NaNO_3$ (Group 3). All the pigs were premedicated by intramuscular ketamine (20 mg/kg), midazolam (0.1 mg/kg), and atropine (0.25 mg) and placed in the supine position. Anesthesia was induced with midazolam (0.1 mg/kg) plus sufentanil (0.5 μg/kg) and was maintained with intravenous sufentanil (0.5 μg/kg/h) and midazolam (0.15 mg/kg/h) infusions. Muscle paralysis was achieved with vecuronium bromide (1 mg/kg) and maintained with vecuronium (2 mg/kg/h) infusions. The lungs were mechanically ventilated via a No. 9 cuffed endotracheal tube (Kendall Curity Tracheal Tube, Tyco Healthcare, Switzerland) with a Servo ventilator 900 C (Siemens, Elema, Sweden). A ventilator was set to deliver forced inspiratory oxygen (F_iO_2) of 0.4, tidal volume between 12 and 15 mL/kg, and respiratory rate adjusted to maintain partial pressure of carbondioxide in arterial blood ($PaCO_2$) in the range of 35 to 40 mm Hg. Positive end-expiratory pressure of 5 cmH_2O was used to prevent atelectasis. Sevoflurane was administered

with a vaporizer adapted to the ventilator. Inspired and expired fractions of oxygen, carbon dioxide, and sevoflurane were measured with an infrared spectrophotometer (Ultima II; Datex, Helsinki, Finland).

Sodium chloride (0.9% at 10 mL/kg/h) infusion via the left internal jugular vein was maintained during surgery. The temperature was adjusted to 38°C to 39°C with the help of an electrical heating pad. Invasive arterial pressure monitoring line catheter was placed into the right common iliac artery for systemic arterial blood pressure and arterial blood sampling. Pulmonary artery catheter (Swan Ganz CCO/VIP; Edwards Lifesciences LLC, Irvine, Canada) was inserted through the right internal jugular vein and positioned under pressure control in a branch of the pulmonary artery for measurement of mean pulmonary arterial pressure (MPAP), central venous pressure (CVP), pulmonary capillary wedge pressure (PCWP), cardiac output (CO), cardiac index (CI), left ventricle end diastolic pressure (LVEDP). ECG was continuously recorded.

Following midline sternotomy, the heart was held in a pericardial tissue. Ultrasonic transit time flow probes were placed around the distal part of the left anterior descending (LAD) artery. A catheter was placed into the proximal part of LAD. This catheter was used both for real time coronary artery pressure monitoring and experimental drug administration. After having blood and tissue samplings (see involved part), same amounts of isotonic sodium (Group 1), $NaNO_2$ (0.5 μg/kg) (Group 2), and $NaNO_3$ (0.5 μg/kg) (Group 3) administered thorough the catheter inside the LAD. All administered drugs were prepared by an anesthesiologist in a transparent syringe with clear color. The surgeon was blinded to the drugs. After a five-minute period following the administration of the drugs, LAD was encircled with 4/0 prolene suture material from the 1/3 distal part and occluded with the help of rubber snare in order to create ischemia. Ischemia was confirmed by morphological cyanosis and ST segment changes in the electrocardiogram. Following 60 minutes of ischemia occlusion was released and 180 minutes of reperfusion period began. At the end of the reperfusion period, pigs were sacrificed by using potassium chloride following the required samplings.

Tissue, Blood Sampling

At four time points (T0, T1, T2, T3) blood samplings and three time points (T1, T2, T3) tissue samplings were performed for alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), creatinine kinase (CK-MB), troponin t (TNT), arterial blood gases, oxidative stress parameters (glutathion peroxidase [GPx], superoxide dismutase [SOD], malondialdehyde [MDA], catalase and nitric oxide [NO_2^-/NO_3^-]).

Tissue samples were taken from the anteroapical part of the left ventricle for both oxidative stress parameter measurement and histopathological examination (light and electron microscopy) at all time points. First sampling from the heart was taken from an area away from the LAD artery in order not to disturb the LAD supplied area. The other samples were taken from the ischemic anteroapical part of the left ventricle.

Blood and tissue samplings time points were as follows: T0: After the insertion of central venos line and arterial catheteterisation; T1: 30 minutes after the midline sternotomy (after a stabilization period); T2: After ischemic period; T3: After reperfusion period.

Blood and Tissue Sample Analysis

The blood samples were collected in glass tubes containing additives and centrifuged at 4°C with 3000 rpm for 5 minutes. Plasma was removed into tubes and stored at –70°C until measurement. Erythrocytes were washed three times with two volumes of isotonic saline. Then, the erythrocytes were lysed with cold distilled water (1 : 4), stored in a refrigerator at 4°C for 15 min., centrifuged at 4°C with 2000 rpm for 15 min. Supernatant were removed and stored at –70°C until measurement. Plasma samples were used for measurement of NO_2^-/NO_3^- and MDA levels. Erythrocyte lysates were used for measuring CAT, GPx and SOD activities. The tissue samples were stored in tubes not containing additives at –70 °C. These samples were homogenized in cold potassium chloride solution (1.15%) in a glass homogenizer and centrifuged at 4°C at 5000 × g. The supernatant was removed and used for GPx and SOD activity, and NO_2^-/NO_3^- and MDA level measurements.

GPx, SOD, nitrite/nitrate, and MDA measurements were performed according to the procedures explained in authors' previous study [4].

Catalase

Erythrocyte CAT activity was measured in hemolysates at 25°C by the method of Aebi. [5] The reaction mixture consisted of 0.05 mmol/l phosphate buffer pH 7.0, 0.01 mmol/l H_2O_2 and erythrocyte lysates. The decomposition rate of the substrate H_2O_2 was monitored spectrophotomet-

rically at 240 nm for 30 sec. The activity was expressed as MU/l. 1 unit is equal to 1 μmol of H_2O_2 decomposed/min.

Histological Examination

Light microscopical study on paraffin sections

Tissue specimens were retrieved, fixed in 10% phosphate buffered formalin (pH 7.0) at room temperature, rinsed in buffer, and dehydrated in a graded series of ethanol before being embedded in paraffin. Five to seven micrometer thick sections were prepared with a rotary microtome (Microm, HM 360, Germany). Haematoxylin & Eosin, and Masson Trichrome stained sections were evaluated for overall cardiac muscle morphology and myocardial recovery. Stained sections (a minimum of ten sections obtained from different levels of each tissue) were examined by at least two independent investigators with a Leica DMR microscope (Germany). The images were captured via Leica DC500 digital camera (Germany).

Light and Transmission Electronmicroscopical Study on Plastic Sections

Tissue specimens were fixed in 2.5 percent glutaraldehyde in Sorensons' phosphate buffer, rinsed in buffer and postfixed in 1 percent osmium tetroxide in PBS at 4°C for 2 hours. Specimens were dehydrated in graded series of ethanols to absolute ethanol in preparation for vacum embedding in araldite Cy 212 (Agar). Semi-thin sections were stained with methylene blue-AzurII; then they were examined and photographed via Leica DMR microcsope and digital camera in a same manner with paraffin sectins. Thin sections were stained with uranyl acetate and lead citrate and documented for subcellular organization of cardiomyocyte by Carl Zeiss-910 model transmisson electron microscope.

Statistical Analysis

SPSS version 15.0 (IL, Chicago, USA) software was used to perform statistical analysis. GLM-repeated measures variance analysis test was used to compare group-timing interactions. Data was expressed as mean ± standard deviation. The Bonferroni test was used as a post hoc analysis to identify the difference between each group after detecting the general difference. A P value of < 0.05 was considered significant.

Results

Hemodynamic Findings

There was no difference between groups for measured hemodynamic parameters at pre-ischemic period ($P > 0.05$). With the beginning of ischemic period there was a decrease in blood pressure, CO and an increase in heart rate, LVEDP and PCWP in all groups. At the reperfusion period, changes in the ischemic period partially returned to their pre-ischemic levels. This return to pre-ischemic levels was most prominent in NO_2--group. While the return to pre-ischemic levels was almost 32 ± 5.2% in NO_3– and control groups, it was bigger than 65% in NO_2– group ($P < 0.05$). The data related with hemodynamic parameters was summarized in Table 1.

Oxidative Stress Parameter Findings

Oxidative stress parameters were both studied at tissue and the blood level. At the blood level there were protective increases with the beginning of the ischemic period at the SOD, GPx, CAT, NO_2^-/ /NO_3^- levels. However, during the reperfusion period, these increases did not continue in control and the $NaNO_3$ groups. In $NaNO_2$ group protective increases persisted also in the reperfusion period. When the groups were compared, there were statistically significant differences in favor of $NaNO_2$ group in T2 and T3 time points (Group 2 T2 and T3 time points for SOD levels: T2: 336.5 ± 74.71, T3: 426.5 ± 56.33 U/ml, $P < 0.001$, GPx levels: T2: 105.16 ± 10.18 U/ml, T3: 132.16 ± 8.3 U/ml, $P < 0.001$, CAT: T2: 309 ± 45.62 U/ml, T3: 359.66 ± 36.34 U/ml, $P < 0.001$, NO_2^-/NO_3^-: T2: 100.16 ± 13.04 μmol/ml, T3: 119.66 ± 8.04 μmol/ml, $P < 0.001$). MDA levels are the cross-check findings for the previous parameters. As a marker of lipid peroxidation MDA levels usually increase due to the cellular damage. In the present study in contrast to the previous parameters, there were statistically significant increases in groups 1 and 3 (Group 1 T2: 3.56 ± 0.13 Group 3: T2: 3.15 ± 0.17, $P < 0.05$ Group 1 T3: 3.55 ± 0.18 nmol/ml, Group 3 T3: 3.3 ± 0.13 nmol/ml, $P < 0.05$). The increase was the most prominent in group I. MDA levels almost stayed normal following an non-significant increase in the post-ischemic period in nitrite group

Table . Hemodynamic parameters

Tabela 1. Wskaźniki hemodynamiczne

Groups (Grupy)	Time (Czas)	Hemodynamic parameters (Wskaźniki hemodynamiczne)							
		MAP mmHg	HR bpm	CO l/min	Cİ l/min/m^2	LVEDP mm Hg	MPAP mm Hg	PCWP mm Hg	CVP mm Hg
Group 1 (Grupa 1)	pre-ischemia	96 ± 4.9	88 ± 7.3	3.9 ± 0.3	2.76 ± 0.2	11 ± 2.4	16 ± 2.8	11 ± 2.6	7 ± 1.7
	post-ischemia	82 ± 6.3*	108 ± 11*	2.7 ± 0.4*	1.91 ± 0.3*	23 ± 3.1*	23 ± 3.1*	18 ± 3.2*	10 ± 2.0*
	post-reperfusion	93 ± 5.0	90 ± 6.6	3.1 ± 0.3	2.19 ± 0.1	14 ± 2.5	18 ± 3.2	14 ± 2.7	8 ± 1.9
Group 2 (Grupa 2)	pre-ischemia	92 ± 4.2	91 ± 6.2	3.9 ± 0.4	2.6 ± 0.2	12 ± 2.0	15 ± 1.9	12 ± 1.9	8 ± 2.1
	post-ischemia	86 ± 4.6*	107 ± 10.3*	3.2 ± 0.2*	2.13 ± 0.2*	20 ± 2.6*	20 ± 1.7*	19 ± 2.8*	11 ± 1.9
	post-reperfusion	90 ± 3.7	89 ± 7.4	3.7 ± 0.3	2.5 ± 0.3	11 ± 2.2	14 ± 1.8	11 ± 2.1	9 ± 0.6
Group 3 (Grupa 3)	pre-ischemia	94 ± 5.1	87 ± 7.6	3.8 ± 0.2	2.6 ± 0.3	10 ± 2.1	17 ± 2.1	10 ± 2.4	7 ± 2.3
	post-ischemia	81 ± 6.1*	110 ± 9.2*	2.8 ± 0.3*	1.93 ± 0.2*	23 ± 2.7*	23 ± 2.0*	17 ± 3.3*	11 ± 2.1*
	post-reperfusion	92 ± 3.,9	90 ± 5.2	3.1 ± 0.2	2.13 ± 0.2	15 ± 2.3	18 ± 2.4	15 ± 2.1	8 ± 1.2

MAP – Mean arterial pressure, HR – Heart rate, CO – Cardiac output, CI – Cardiac index, LVEDP – Left ventricle enddiastolic pressure, MPAP – Mean pulmonary artery pressure, PCWP – Pulmonary capillary wedge pressure, CVP – Central venous pressure, bpm – beat per minute.
* $P < 0.05$.

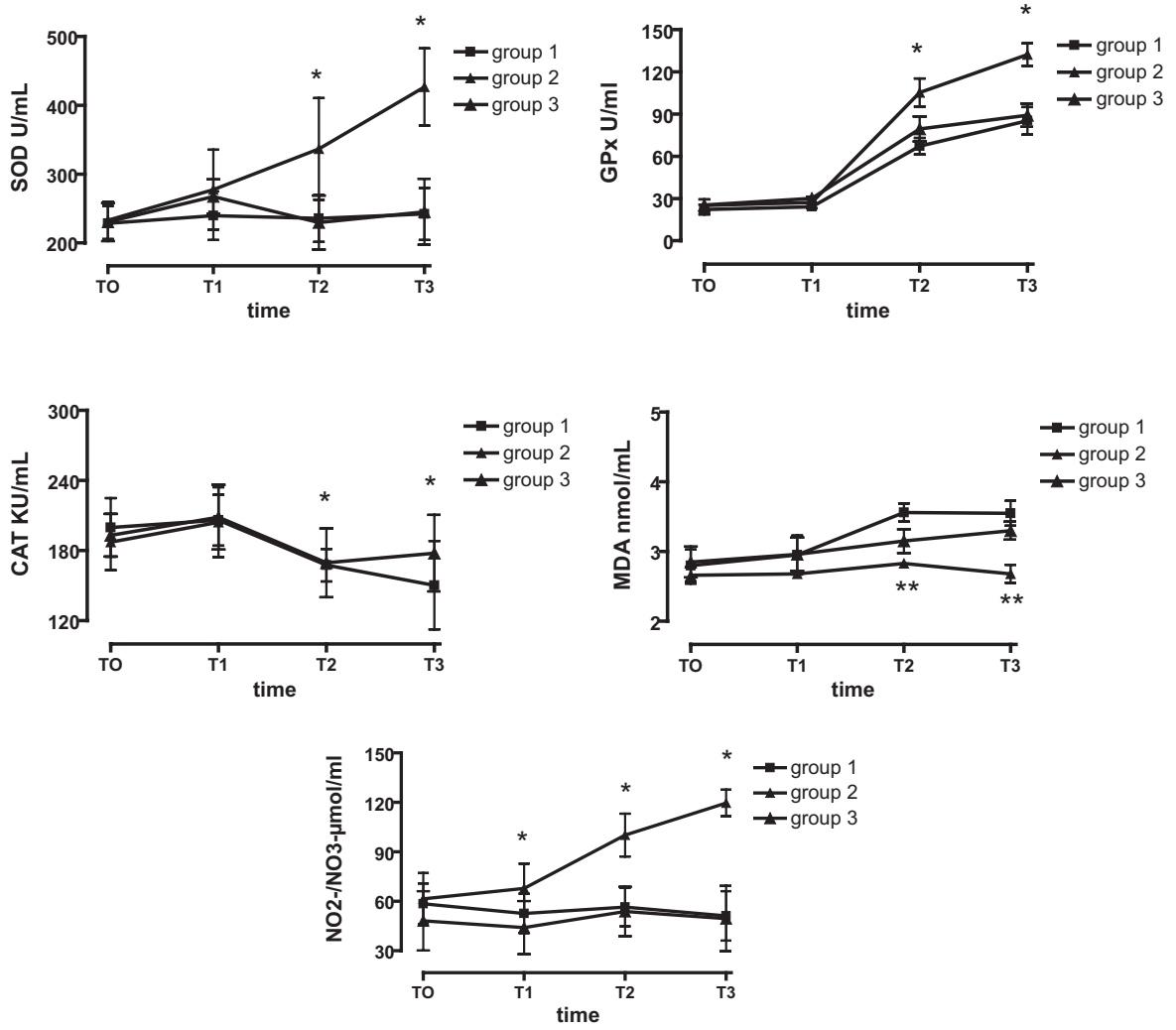
MAP – średnie ciśnienie tętnicze, HR – tętno, CO – rzut serca, CI – wskaźnik sercowy, LVEDP – lewokomorowe ciśnienie końcowo-rozkurczowe, MPAP – średnie ciśnienie w tętnicy płucnej, PCWP – ciśnienie zaklinowania, CVP – ośrodkowe ciśnienie żylne, bpm – uderzeń na minutę.
* P < 0,05.

(Group 2 T2: 2.83 ± 0.17 nmol/ml, T3: 2.68 ± 0.13 nmol/ml). When these results were compared with group 1 and 3, there were significant differences in favor of NO_2– group (Group 2 vs Group 1:$P <$ < 0.001, Group 2 vs. Group 3:$P < 0.05$). The changes in four different time points in all studied parameters were shown in Fig. 1.

Tissue level findings were also similar to the blood level findings. While there were protective increases in post-ischemic and post-reperfusion measurements in SOD, GPx levels, this was the most prominent in NO_2– group (SOD: T1: 360.12 ± 38.55 U/g T2: 380.33 ± 119.72 U/g [Group 2 vs Group 1 and Group 2 vs Group 3, $P < 0.05$], GPx: T1: 101.16 ± 10.59 U/g T2: 172.83 ± 31.22 U/g [Group 2 vs Group 1: $P < 0.05$, Group 2 vs. Group 3: $P < 0.001$]). The changes were different for CAT, NO_2^-/NO_3^- levels. With the beginning of the ischemic period there was no increase in these parameters different form the blood levels. The only increase was in the NO_2 – group in CAT levels (T1: 324.33 ± 44.96 U/g, T2: 403,33 ± 34.65 U/g). There were significant differences from group 1 and group 3 in favor of group 2 (Group 2 vs. Group 1 and Group 2 vs. Group 3: $P < 0.001$). There was no difference between group 1 and group 3 ($P = 1.00$). For NO_2^-/NO_3^- levels, while there was a progressive increase in nitrite group, there was a decrease in group 1 and a near decrease in group 3. The difference again was in favor of group 2 ([T1: 2.48 ± 1.9 μmol/g, T2: 4.57 ± 3.51 μmol/g] [Group 2 vs. Grup 1 and Group 2 vs. Group 3: $P < 0.05$]). The difference between group 1 and group 3 was not significant ($P = 0.888$). Changes in the tissue MDA levels were very similar in group 1 and group 3 ($P = 0.303$). There was a progressive increase in this parameter. In group 2 following a slight increase in the post-ischemic period the levels returned to baseline levels. When groups compared, again there was significant change in favor of NO_2– group ([T1: 1.88 ± 0.98 nmol/g, T2: 1.3 ± 0.56 nmol/g] [Group 2 vs. Group 1 and Group 2 vs. Group 3: $P < 0.05$]). The changes in the tissue level examinations were summarized in Fig. 2.

Cardiac Enzyme Findings

There was no statistically significant difference in any timepoint for ALT assesment. There was only a significant difference for T3 measurement in favor of nitrite group when compared to other groups for AST ($p < 0.05$), LDH (Group 2 vs. Group 1 $P < 0.01$, Group 2 vs Group 3 $p > 0.05$), CK-MB (Group 2 vs. Group 1 $P < 0.001$, Group 2 vs. Group 3 $P = 0.012$) and TNT levels (Group 2 vs. Group 1 and Group 1 vs. Group 3: $P < 0.05$, Group 2 vs. Group 3 $P > 0.05$). The changes in cardiac enzyme levels were shown at Fig. 3.



* $p < 0.05$, **$p < 0.001$
SOD – superoxide dismutase, GPx – Glutathion peroxidase, CAT – Catalase, MDA – Malondialdehyde

Fig. 1. Oxidative parameter changes in blood level

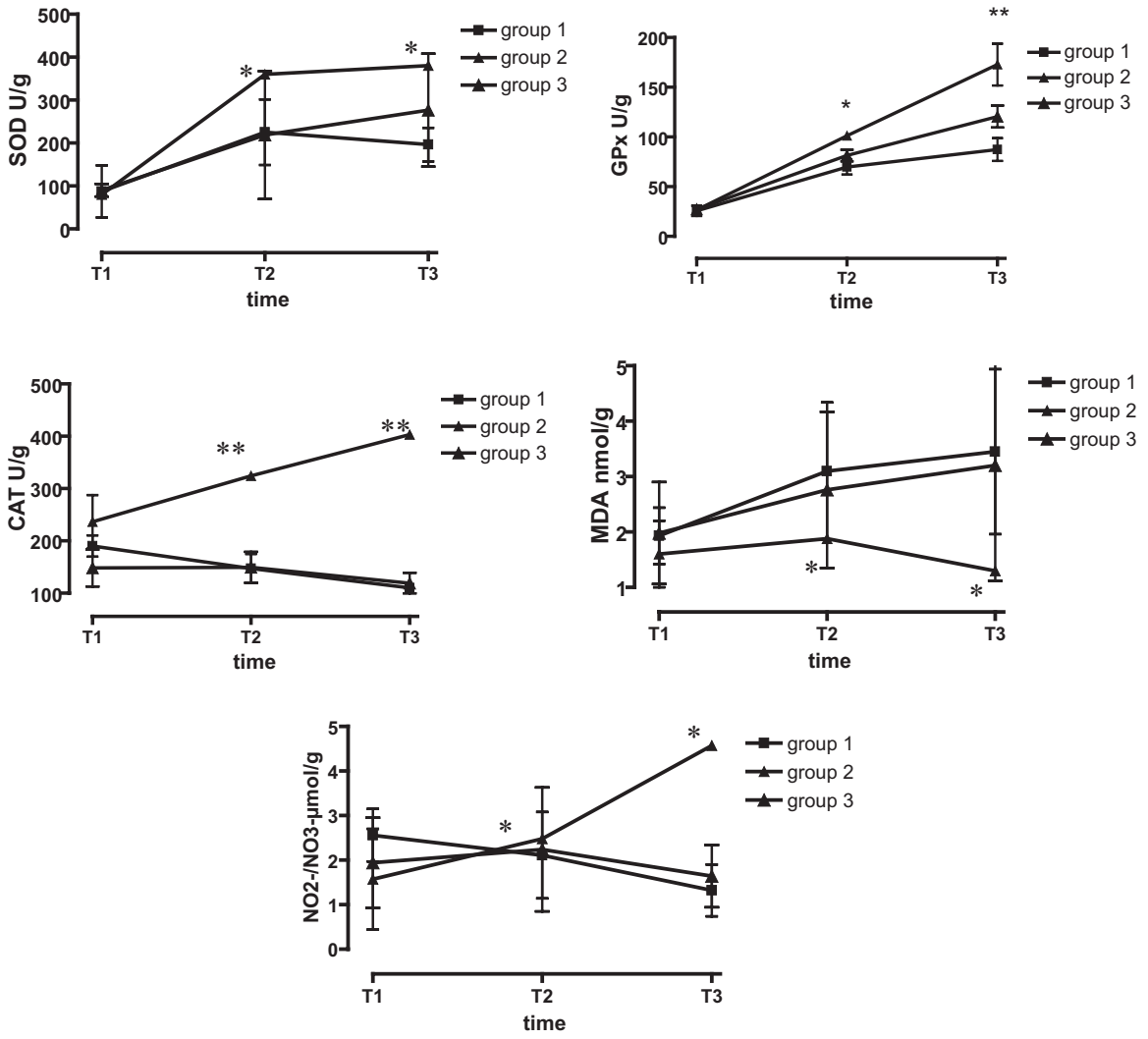
Ryc. 1. Zmiany stężenia wskaźników oksydacyjnych krwi

Histological Findings

Tissue samples that were isolated from the same region of the pig heart were assessed for myocardial recovery at light and transmission electron microscopically. Cardiomyocyte cytoplasm stains pinkish with haematoxylin & eosin; red with Masson's trichrome and blue with methylene blue azur II. Collagen fibers stain green with trichrome (Figs. 4–6).

Control and NO_2– groups' micrographs revealed vascular congestion and local edema following ischemia and reperfusion (Fig. 4D–F). Mononuclear cellular infiltrates and some adipocytes were noted after ischemia and reperfusion in NO_2– group (Fig. 4B). On the other hand, the junctional complexes (intercalated discs) were intact in this group (Fig. 4E, F). The sarcomere alignment, mitochondria and nuclei were better preserved within the myocyte with NO_2– treatment comparing to control and NO_3– groups electron microscopically. Crista deterioration was noted at the post-ischemic period with nitrite treatment. Otherwise, the ischemia reperfusion injury was considered severe in all groups because of the absence of glycogen adjacent to the mitochondria. On the other hand, in NO_2– group mitochondria exhibited intact double membranes, compact, relatively organized cristae and a homogeneous dense matrix after reperfusion (Figs. 7A–F, 8A–F).

The myocyte cytoplasms appeared depleted (Fig. 6E, F) and vascular congestion was noted with the presence of mononuclear cell infiltrates light microscopically in NO_3-group (Fig. 6A–F). Depleted nuclei and giant, depleted mitochondria,



* p < 0.05, **p < 0.001
SOD – superoxide dismutase, GPx – Glutathion peroxidase, CAT – Catalase, MDA – Malondialdehyde

Fig. 2. Oxidative parameter changes in tissue level

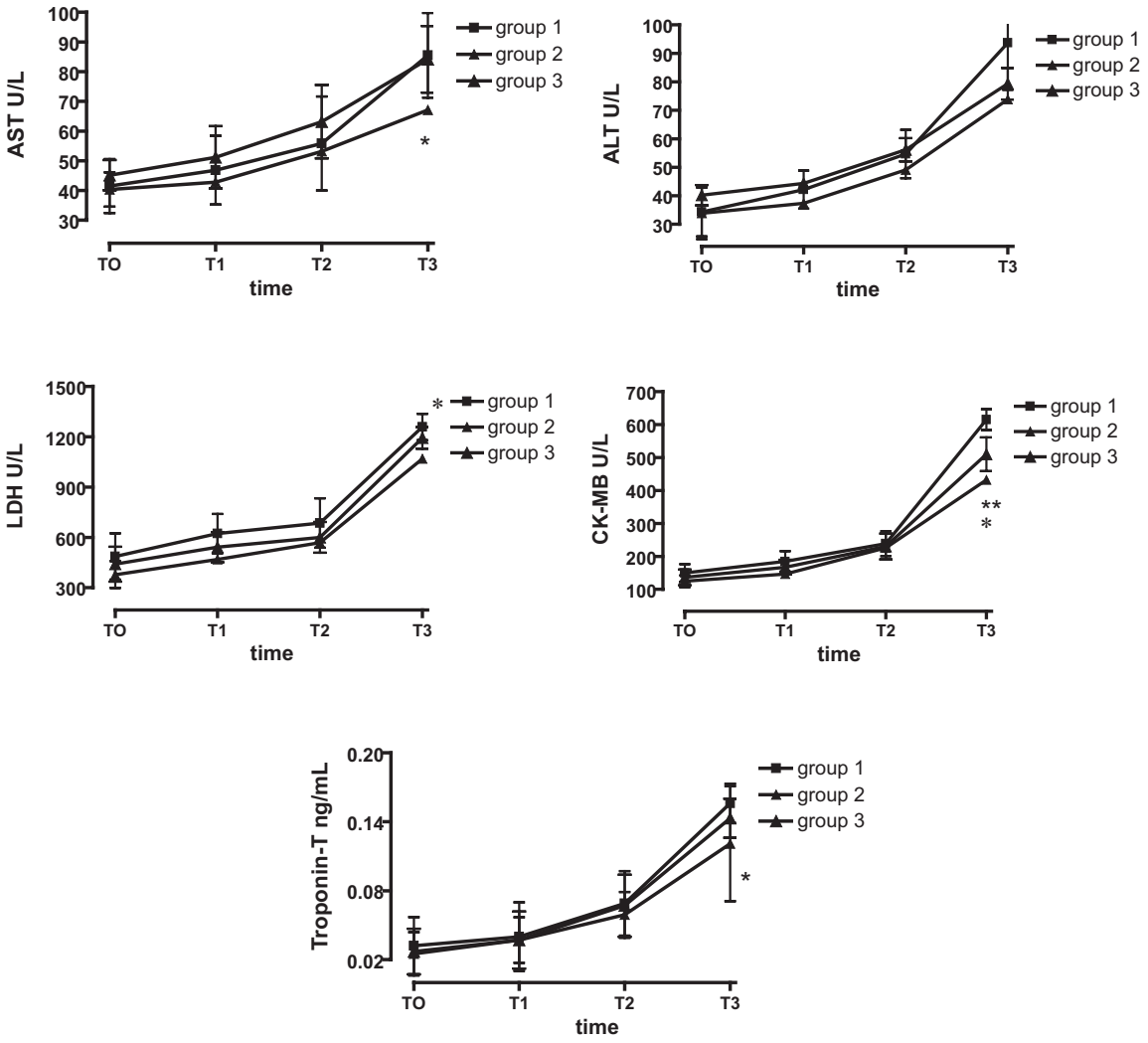
Ryc. 2. Zmiany stężenia wskaźników oksydacyjnych w tkankach

leaving open spaces between them were noted with an electron microscope after ischemia and reperfusion with NO_3– treatment (Fig. 8A–F). Mitochondrial membranes were disrupted and exhibited dense bodies intervening irreversible reperfusion injury. Control group also exhibited condensed mitochondria with abnormal cristae following reperfusion; although the microstructure was slightly better preserved compared to that of NO_3– group (Fig. 8E, F).

Discussion

The present data indicates that exogenous nitrite administration just before ischemia protects the pig heart against damage from ischemia and reperfusion injury. The reductive conversion of NO_2– to NO is thought to occur by a number of mechanisms including the enzymatic actions of xanthine oxidoreductase [6], non-enzymatic disproportionation [7] and a hemoglobin reductase activity that is under allosteric control [8]. These mechanisms of NO2– reduction favor bioconversion of NO_2– to NO under the hypoxic and acidic conditions present during ischemia [9].

Gonzalez wrote: "During cardiac ischemia and reperfusion, NO_2– in tissue is reduced to NO and forms ironnitrosylated (Fe^{+2}–NO) and S-nitrosated modified proteins, via reactions with deoxymyoglobin and other cellular heme proteins. The rapid, facile metabolism of NO_2– to NO with sub-



* p < 0.05, **p < 0.001
ALT – alanine aminotransferase, AST – aspartate aminotransferase, LDH – lactate dehydrogenase, CK- MB – creatinine kinase, TNT – troponin t

Fig. 3. Changes in cardiac enzymes

Ryc. 3. Zmiany enzymów sercowych

sequent modification of target proteins has been documented in the heart and liver during both regional and global IR injury. The formation of NO in the heart during ischemia has been documented using electron paramagnetic resonance and liquid and gas phase chemiluminescence. The authors have recently been found that NO_2– will specifically posttranslationally S-nitrosate complex I of the mitochondrial electron transport chain (METC); this effectively reduces electron flow through the METC and reduces reactive oxygen species formation during reperfusion. This damping or tuning of electron transport inhibits opening of the mitochondrial permeability transition pore, decreases cytochrome C release, and limits apoptosis. The nitrite-dependent decrease in TUNNEL staining is consistent with this mechanism of cytoprotection. Other intracellular targets for S-nitrosilation by NO_2– during I/R exposure could include the L-type calcium canal receptor. In addition, stabilization of myglobin as iron-nitrosylated myoglobin may limit heme based oxidation reactions in the cardiomyocyte" [9].

In the current study, while there were protective increases in oxidative parameters (tissue and serum) as well as hemodynamic and histopathological examinations in favor of NO_2– group, these changes were not detected in NO_3– group. These findings suggest the cardioprotective effects of $NaNO_2$.

With the beginning of the ischemic period in all groups, there was a decrease in arterial blood

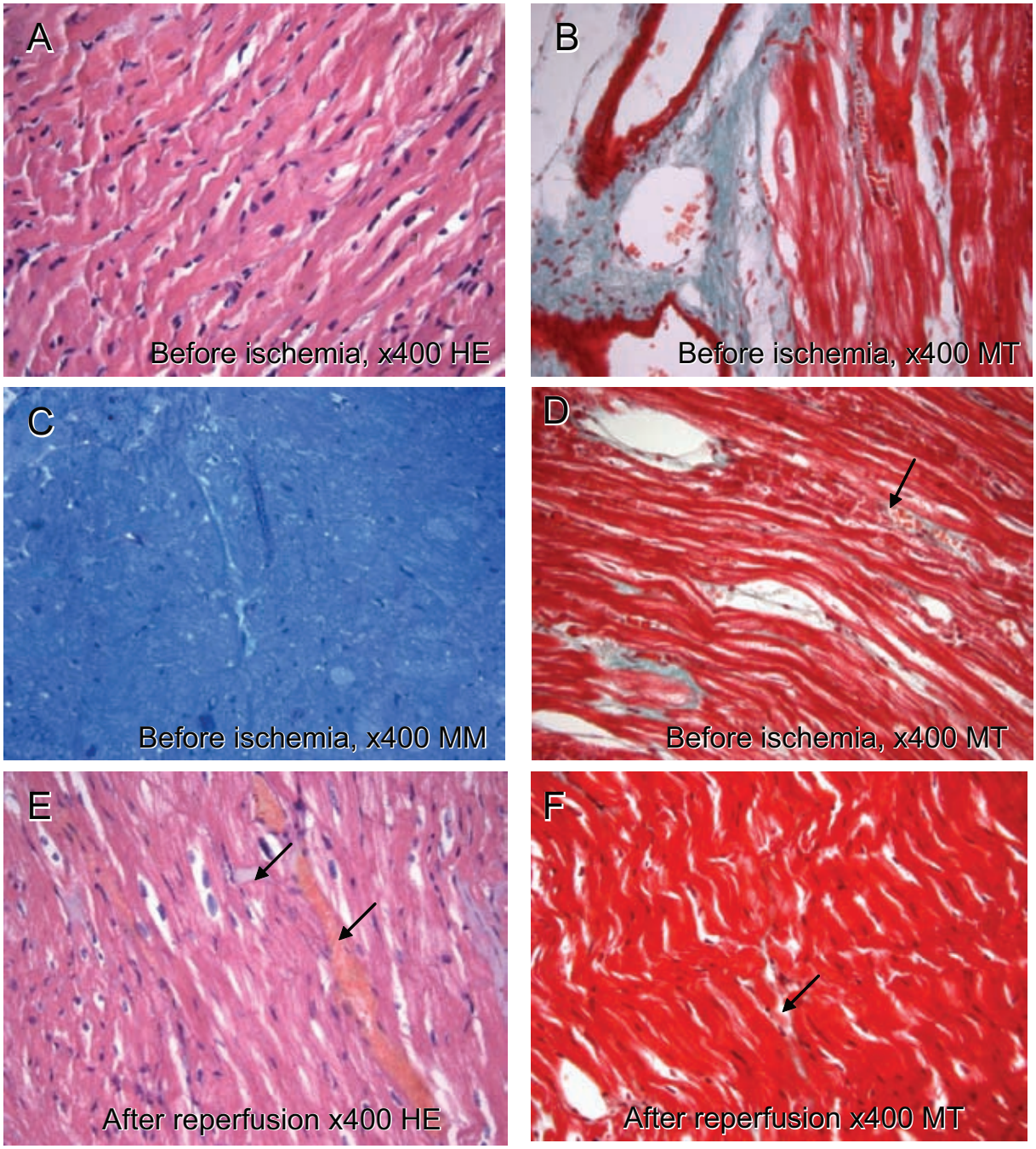


Fig. 4. In control group micrographs, cytoplasms of cardiomyocytes are pink at A and E, red at B, D and F and blue in color at the C. Collagen fibrils in the connective tissue have green color with MT. At D–F congestion at the vessels and minimal edema (arrows) are noted. HE – Hematoxylin-Eosin, MT – Masson's Trichrome, MM – Methylene blue

Ryc. 4. Na mikrofotografii A i E w grupie kontrolnej cytoplazma kardiomiocytów zaznaczona na różowo, B, D i F na czerwono, a C na niebiesko. Włókna kolagenu w tkance łącznej w MT są zielone. W D–F widać przekrwienie w naczyniach i minimalny obrzęk (strzałki). HE – hematoksylina-eozyna, MT – trichromian Massona, MM – błękit metylenowy

pressures and increase in the heart rates. Following the reperfusion period these changes returned to their pre-ischemic levels. Also, there was a decrease in cardiac output measurements in all groups. The decrease returned to pre-ischemic level more than 65% in NO_2– group, while this returning level was 35 ± 5.2% in the other groups ($P < 0.01$). There were also parallel changes in LVEDP measurement. Return in LVEDP level in NO_2– group was more than > 62%, and 32 ± 7.4% in the other groups ($P < 0.05$). There were no statistically meaningful changes between groups for the parameters of CVP, MPAP and SVRI. These findings were in accordance with the findings of Rassaf et al. [10] who concluded that "inhibition of endogenous NO release reduces, whereas replen-

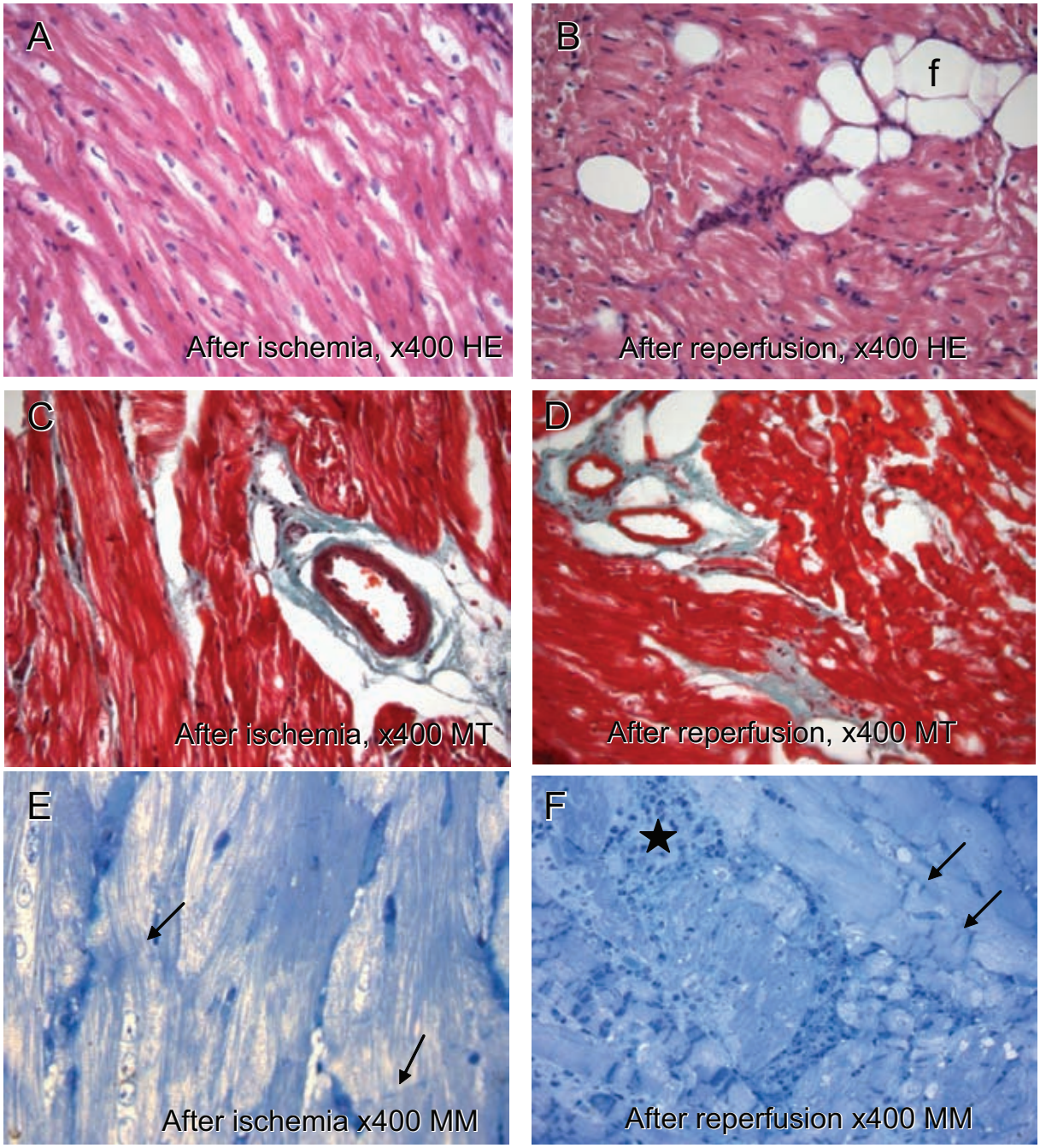


Fig. 5. In nitrite groups micrographs, cytoplasms of cardiomyocytes appear pink at A and B, red at C, and D and blue in color at the E and F. Collagen fibrils in the connective tissue appear green with MT. Fat cells(f) are noted in B. There is local edema between cells in D. Protected intercalated discs (arrow) are noted after ischemia and reperfusion in E and F. Note the presence of mononuclear cellular infiltration near the cardiomyocytes. HE – Hematoxylin-Eosin, MT – Masson's trichrome, MM – Methylene blue

Ryc. 5. Na mikrofotografii A i B w grupie azotynów cytoplazma kardiomiocytów zaznaczona na różowo, C i D na czerwono oraz E i F na niebiesko. Włókna kolagenowe w tkance łącznej są zielone w MT, B – widoczne komórki tłuszczowe (f), D – widać miejscowy obrzęk między komórkami, E i F – widoczne interkalowane płytki (strzałka) po niedokrwieniu i reperfuzji. Należy zwrócić uwagę na infiltrację komórek jednojądrzastych w pobliżu kardiomiocytów. HE – hematoksylina-eozyna, MT – trichromian Massona, MM – błękit metylenowy

ishment with exogenous NO increases LV function, pointing towards a positive effect of tonically released NO on LV function in healthy humans".

Gonzalez et al. [9] in their study investigated "the effect of efficacy of NO_2– in reducing necrosis and apoptosis in canine myocardial infarction and to determine the relative role of NO_2–. They found that nitrite improved microvascular perfusion, reduced apoptosis, and improved contractile function. The findings of the

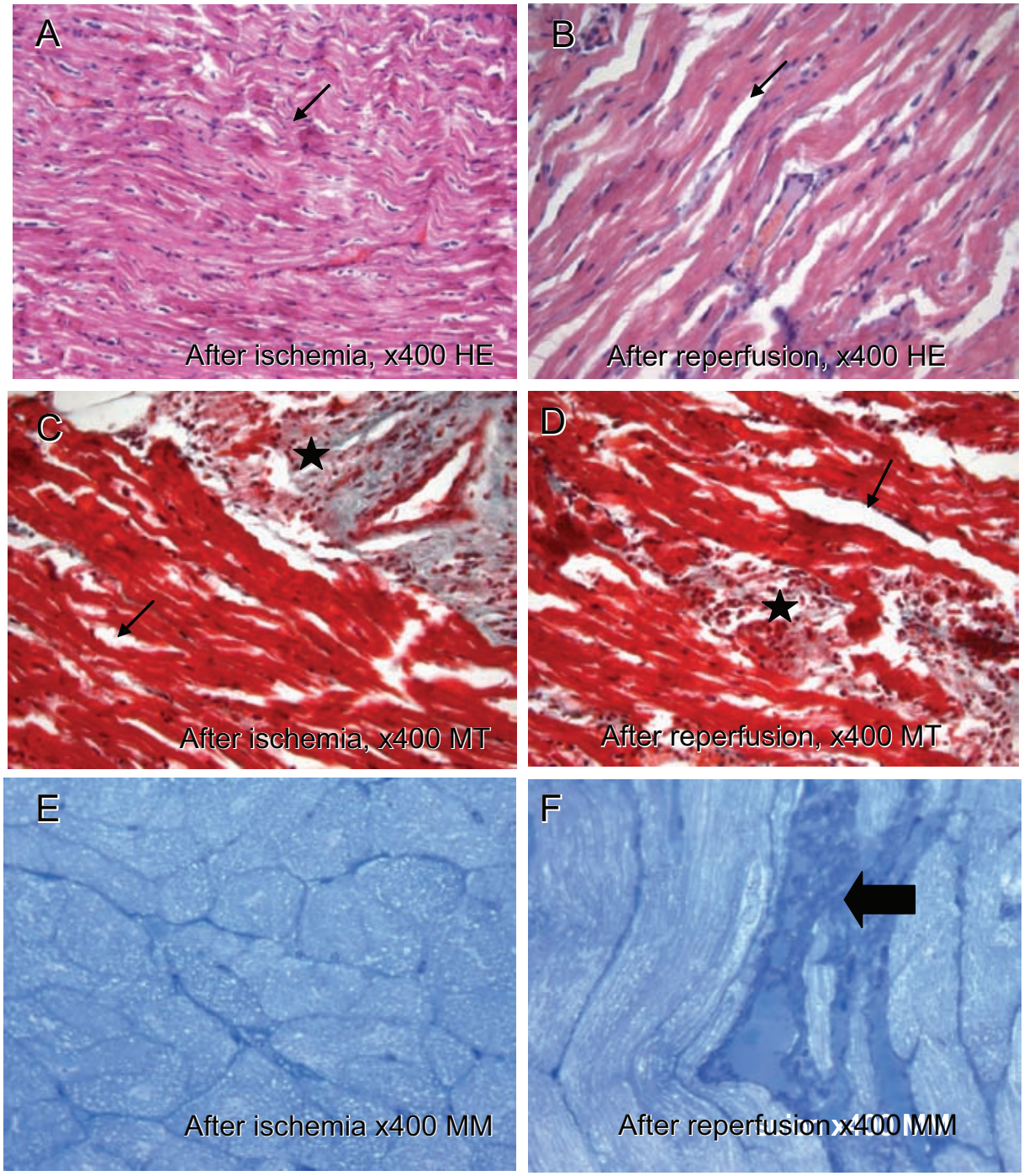


Fig. 6. In the nitrate group micrographs the cytoplasms of cardiomyocytes are pink at A and B, red at C, and D and blue in color at the E and F. Collagen fibrils in the connective tissue appear green with MT. At A–D (arrow) the myofibrils were separated from each other due to edema. Myocyte cytoplasms were partially depleted at E and F after ischemia and reperfusion. At F vascular congestion (wide arrow) was noted between the cells and the junctional complexes cannot be detected. Note the mononuclear cellular infiltration near the cardiomyocytes at C, D.
HE – Hematoxylin-Eosin, MT – Masson's trichrome

Ryc. 6. Na mikrofotografii A i B w grupie azotanów cytoplazmy kardiomiocytów zaznaczono na różowo, C i D na czerwono oraz E i F na niebiesko. Włókna kolagenowe w tkance łącznej są zielone w MT. A–D (strzałka), C i D – miofibryle zostały oddzielone od siebie z powodu obrzęku, E i F – cytoplazma kardiomiocytów została częściowo wyczerpana po niedokrwieniu i reperfuzji, F – widać zator naczyniowy (szeroka strzałka) między komórkami i nie można wykryć połączenia międzykomórkowego złożonego, C, D – należy zwrócić uwagę na infiltrację komórek jednojądrzastych w pobliżu kardiomiocytów. HE – hematoksylina-eozyna, MT – trichromian Massona

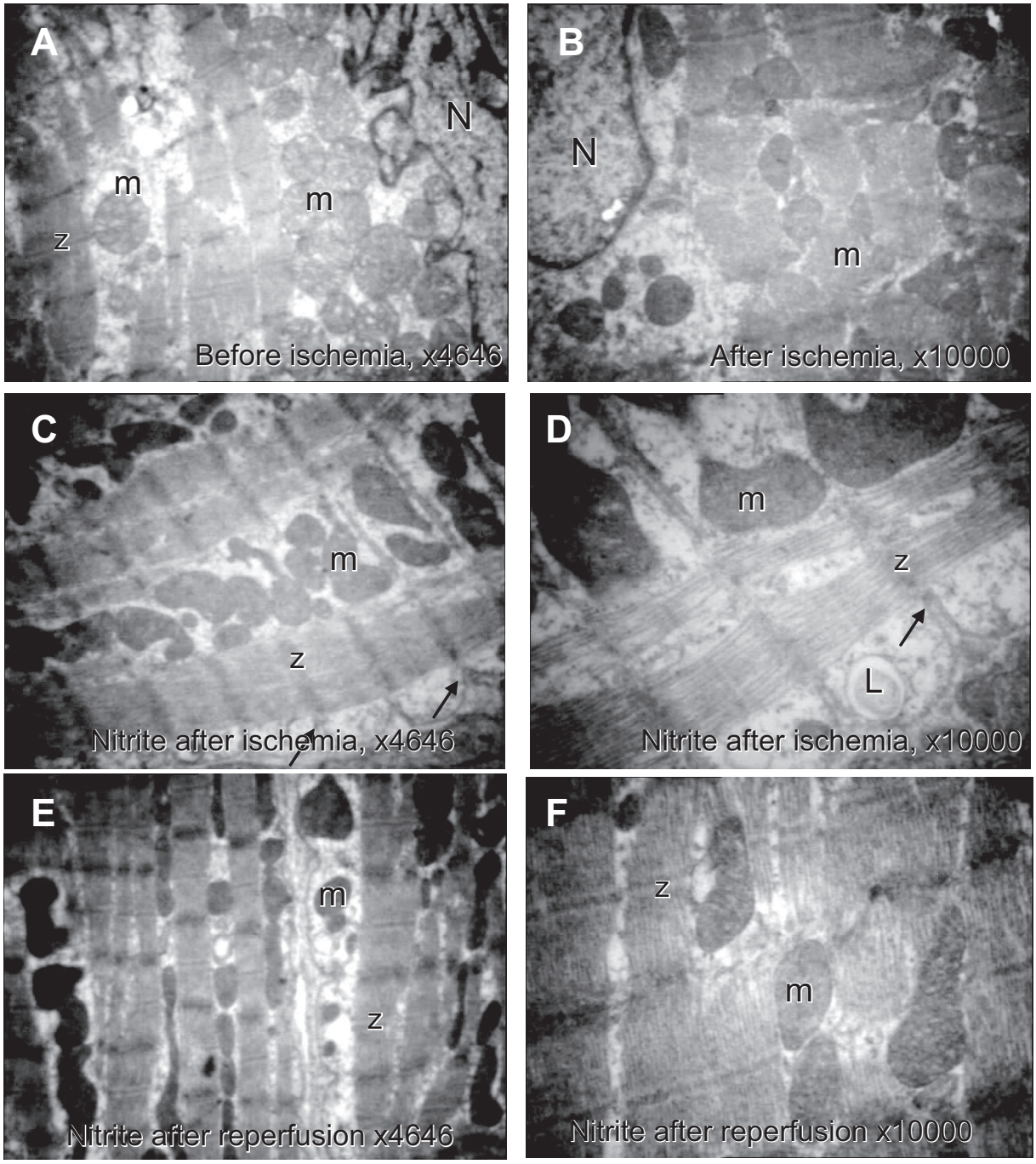


Fig. 7. In the control and nitrite groups electron micrographs, the nuclei (N) and the mitochondria (m) appear healthy in A. A mitochondrial cristae deterioration was noted in control and the nitrite groups after ischemia (B–D). At F the cardiomyocyte exhibits intact mitochondrial membranes and also intact contractile apparatus after reperfusion with nitrite. Z – z band. Uranyl acetate and lead citrate

Ryc. 7. Na zdjęciach wykonanych mikroskopem elektronowym w grupie kontrolnej i w grupie azotynów jądra (N) oraz mitochondria (m) wyglądały prawidłowo (A). Pogorszenie grzebieni mitochondrialnych odnotowano w grupie kontrolnej i grupie azotynów po niedokrwieniu (B–D). Na zdjęciu F kardiomiocyty wykazują nienaruszone błony mitochondrialne, a także nienaruszony aparat skurczowy po reperfuzji za pomocą azotynów. Z – octan uranylu i cytrynian ołowiu

present study are also consistent with the results of this study".

When changes in the oxidative parameters following ischemia and reperfusion period are evaluated, enzyme systems such as superoxide dismutase, glutathione peroxidase, catalase play important role. In the present study there were significant protective increases in SOD, GPx and CAT levels that protect myocardium from ischemia/reperfusion injury in NO_2– group when

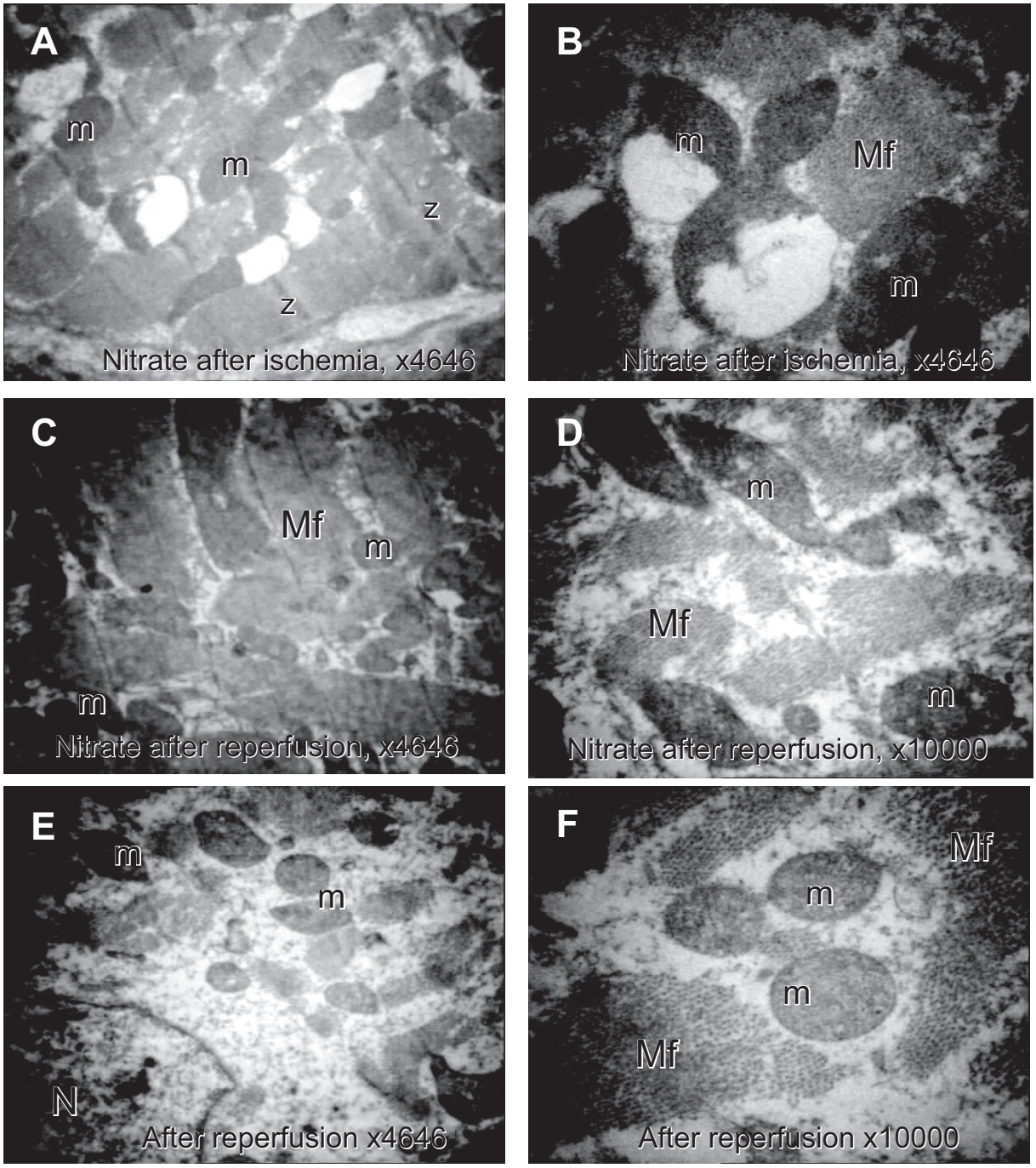


Fig. 8. The control nitrate groups electron micrographs are presented. At A and B giant mitochondria (m) with deteriorated cristae and disrupted membranes are noted after ischemia with nitrate. At C–F mitochondria with condensed and reduced cristae adjacent to myofilaments (Mf) are observed. The nucleus (N) is damaged but still exhibit an continuous membrane in E. Uranyl acetate and lead citrate

Ryc. 8. Zdjęcia wykonane mikroskopem elektronowym w grupie kontrolnej i w grupie azotanów; A i B – można zauważyć gigantyczne mitochondria (m) ze zdegenerowanym grzebieniem i przerwanymi błonami po niedokrwieniu z użyciem azotanów, C–F – widać mitochondria z zagęszczonymi i zmniejszonymi grzebieniami w sąsiedztwie włókien (Mf), E – jądro (N) jest uszkodzone, ale nadal ma ciągłą błonę. Octan uranylu i cytrynian ołowiu

compared to other groups ([SOD serum level $P < 0.001$, tissue level $P < 0.001$], [GPx serum level $P < 0.001$, tissue level $P < 0.001$], [CAT serum level $P < 0.001$, tissue level $P < 0.001$]).

Endothelial cells are activated in the reperfused myocardium and they express adhesion proteins, secrete cytokines and decrease NO production. As a result of this process, neutrofills and monocytes accumulate and are activated in ischemic-reperfused tissue. Release of reactive oxygen species and proteolytic enzymes from these activated cells may damage myocyte and vascular cells [4, 11]. High

levels of NO inhibit these processes during reperfusion. In this study serum and tissue levels of NO_2^-/NO_3^- were statistically higher in nitrite group from the other groups (Serum levels: Group 1: $P < 0.001$, Group 3: $P < 0.001$, Group 1–3: $P = 0.466$), (Tissue levels: Group 1: $P < 0.05$, Group 3: $P < 0.05$, Group 1–3: $P = 0.888$).

Markers that are used to detect the myocyte damage (AST, ALT, LDH, CK-MB, Troponine-t) were also investigated in the study groups. In the four different sampling points there were only significant changes in ALT and AST levels at the reperfusion period (T3) in favor of nitrite group. There was no significant change in LDH levels in neither group at all time points. The reason for this may be the shortness of the experiment period. There were also no significant changes in CK-MB and troponine-t levels at T0, T1, and T2 time points between groups. However, there was a statistically significant difference in T3 time point in favor NO_2– and NO_3– groups when compared to control group. When both medicated groups compared to each other measured levels of CK-MB was significantly less in NO_2– group ($P = 0.012$). There were also parallel changes in troponine-t levels. These findings also support the cardioprotective value of $NaNO_2$.

Intact junctional complexes (intercalated discs) at light microscopy in NO_2– group and better sarcomere alignment, mitochondria and nuclei were preserved within the myocyte with NO_2– treatment at the electron microscopy also support the findings of oxidative parameters and cardiac destruction enzymes and they are the visual evidence of these findings.

As a conclusion, findings of the present study support the cardioprotective role of $NaNO_2$ against myocardial ischemia-reperfusion injury when it is given at the pre-ischemic period.

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