Mildronate, a Novel Fatty Acid Oxidation Inhibitor and Antianginal Agent, Reduces Myocardial Infarct Size Without Affecting Hemodynamics

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Abstract: Mildronate is a fatty acid oxidation inhibitor approved as an antianginal drug in parts of Europe. We carried out the first study to determine whether a 10-day course of mildronate could reduce myocardial infarct size (IS) during acute myocardial ischemia. Sprague Dawley rats received 200 mg/kg/d of mildronate (treated group, n = 16) or sterile water (control group, n = 14) subcutaneously for 10 days before ischemia-reperfusion. Rats were then subjected to 45 minutes of left coronary artery occlusion and 2 hours of reperfusion. The 2 groups had identical areas at risk: treated 38 ± 3%; controls 38 ± 2%. The amount of necrosis was smaller in the mildronate group at 16 ± 2% of the left ventricle versus controls, 22 ± 2% ($P = 0.05$); and for any amount of risk > 25%, necrosis was smaller in the treated group ($P = 0.0035$). Myocardial IS (% of risk zone) was 43 ± 3% in the mildronate-treated rats, and 57 ± 4% in controls ($P = 0.004$). During occlusion, there were no differences between the 2 groups in heart rate (216 ± 12 bpm, mildronate and 210 ± 9 bpm, control), in mean arterial pressure (60 ± 2 mm Hg, mildronate and 64 ± 3 mm Hg, control) or in the frequency of arrhythmias. Our study for the first time demonstrated that a 10-day treatment with mildronate reduced myocardial IS in an experimental model of acute myocardial ischemia, without any effect on hemodynamics.

Key Words: fatty acid oxidation, myocardial infarction, infarct size

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Acute myocardial ischemia, which is caused by a critical imbalance between oxygen supply and oxygen demand in the heart, develops in myocardial infarction when the blood supply to an area of the myocardium is reduced as a consequence of partial or complete occlusion of a blood vessel. Despite the extensive use of thrombolysis and percutaneous coronary interventions, both the morbidity and mortality in patients suffering from myocardial ischemia and myocardial infarction remain considerable. Pharmacological interventions that could correct the imbalance between oxygen supply and demand have the potential to further benefit outcome in such patients. One of the less exploited therapeutic possibilities is so called metabolic correction, in which cardiac metabolism is shifted toward glucose utilization during ischemia. A shift toward increased glucose utilization could be accomplished by inhibiting the oxidation of free fatty acids.[1] On the basis of this assumption, several inhibitors of fatty acid oxidation have been developed. Some of them (ie, trimetazidine and mildronate) are available for clinical use to treat angina pectoris.[2] However, it is unknown whether treatment with a course of mildronate could limit myocardial infarct size (IS). Limiting the size of the myocardial infarction has been suggested to be an important clinical goal.[3] Therefore, new therapies that reduce the extent of necrosis and provide improved cardioprotection in patients with myocardial infarction are needed. In the current study, we focused our attention on a new antianginal agent that inhibits fatty acid oxidation—mildronate.

Mildronate [3-(2,2,2-trimethylhydrazinium) propionate] was developed in 1988 by a group of scientists from the Latvian Institute of Organic Synthesis. This agent was shown to inhibit γ-butyrobetaine hydroxylase (GBBH), the enzyme that catalyzes carnitine biosynthesis from γ-butyrobetaine in the liver[4] (Fig. 1). Several studies that followed have shown the cardioprotective effect of mildronate against cardiac dysfunction induced by hypoxic conditions, and ischemia and reperfusion.[5–10] We hypothesized that its antianginal properties could translate into a reduction of ischemic necrosis in the heart during acute myocardial ischemia. Therefore, we carried out the first study to determine whether a 10-day course of mildronate could reduce myocardial IS during acute myocardial ischemia, by using an experimental rat model of coronary occlusion and reperfusion. A 10-day course was chosen to mimic the situation of a patient receiving the drug chronically for angina.

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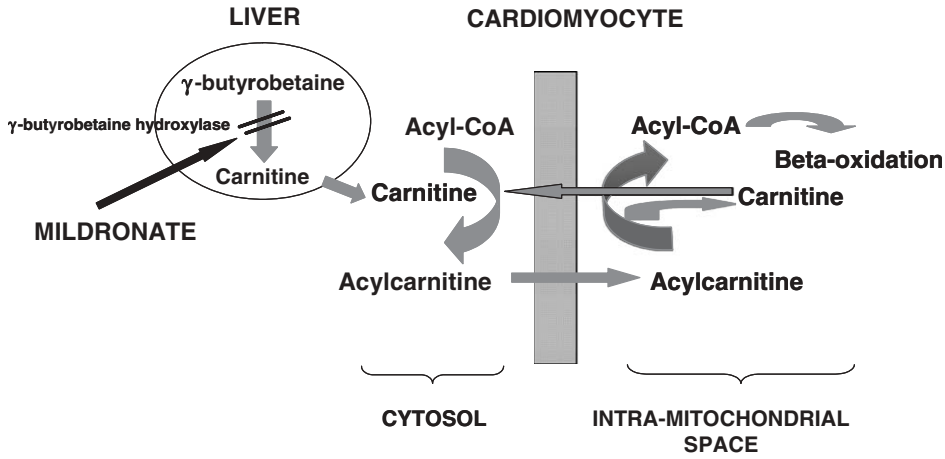

FIGURE 1. Mildronate inhibits liver GBBH, and decreases carnitine biosynthesis from γ-butyrobetaine. Decreased biosynthesis of carnitine reduces its concentration in the heart, and consequently diminishes the biosynthesis of long-chain acylcarnitine in the cytosolic compartment of the cardiomyocytes. Decreased biosynthesis of long-chain acylcarnitine in the cytosol reduces its concentration in the intramitochondrial space. Accordingly, a smaller amount of long-chain acyl-CoA is formed inside the mitochondria, and less long-chain acyl moieties enter the β-oxidation cycle. Thus, mildronate's ability to effectively reduce concentration of long-chain acylcarnitine and long-chain acyl-CoA translates into the inhibition of fatty acid β-oxidation.

METHODS

Animals

Sprague Dawley female rats (200 to 300 g) were maintained in accordance with the policies and guidelines of the Position of the American Heart Association on research animal use[11] and the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (Department of Health, Education and Welfare Publication No. 85-230). Animal procedures were approved by the Institutional Care and Use Committee at Good Samaritan Hospital.

Rationale of the Study

A 10-day pretreatment with mildronate was chosen to optimize the biochemical effect of the drug, that is, to reduce carnitine to a level that would have resulted in inhibition of fatty acid β-oxidation. In previous studies, we demonstrated that mildronate given at a dose of 150 mg/kg orally for 10 days reduced rat heart carnitine level by 63.7%.[4]

Treatment

Rats were randomly divided into 2 groups: control and mildronate-treated. The mildronate group (n = 16) received a daily dose of 200 mg/kg of mildronate (Institute of Organic Synthesis, Riga, Latvia), subcutaneously for 10 days, until the day before undergoing myocardial ischemia-reperfusion. For subcutaneous injections the drug was dissolved in sterile water. The control group (n = 14) received an equivalent volume of sterile water (vehicle), subcutaneously for 10 days. Before starting the protocol, we performed a *tolerability test* on a separate group of rats to determine the best tolerated route of administration (intraperitoneal or subcutaneous). Subcutaneous administration did not cause any rash or irritation in the skin of the rats nor did it induce any other side effects.

Myocardial Ischemia-reperfusion

On the 11th day (after the 10-day treatment), the rats were anesthetized with an intraperitoneal injection of 75 mg/kg ketamine and 5 mg/kg xylazine, shaved, endotracheally intubated, and ventilated with a rodent respirator. Their chests and necks were swabbed with betadine and then alcohol. A cut-down was made in the neck to expose the carotid artery and the jugular vein. Fluid-filled catheters were inserted into the jugular vein to administer fluids and into the carotid artery. A pressure transducer connected to the carotid artery-catheter was used to measure heart rate and arterial pressures. Body (rectal) temperature was measured through a probe inserted into the rectum and maintained using a heating pad.

A left thoracotomy in the fourth intercostal space was performed and the pericardium removed. A 4-O silk suture was placed under the proximal portion of the left coronary artery; the ends of the suture were threaded through a piece of plastic tubing, forming a snare that occluded the artery when tightened and clamped. The coronary artery was occluded (CAO) for 45 minutes, and then reperfused (by releasing the clamp) for 2 hours. Additional anesthesia was given during the study as required. At the end of the 2 hours of reperfusion, the coronary artery was reoccluded, and 0.7 mL of a 50% solution of Unisperse blue dye (Ciba-Geigy, Hawthorne, NY) was injected via the jugular vein to identify the ischemic risk area (the area that did not receive the blue dye, because it was not perfused). With the rats under

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deep anesthesia, 2 mL of potassium chloride (2 mEq/mL) was injected into the jugular vein to stop the heart in diastole and the heart was excised.

Hemodynamics

Heart rate, mean arterial pressure, systolic blood pressure, and diastolic blood pressure were recorded through a pressure transducer (ADInstruments, Colorado Springs, CO) at different time points in the course of the experiment [before CAO (ie, baseline), at 45 min during CAO, and at 1 and 2 h during reperfusion]. Arrhythmias were documented from heart rate and blood pressure tracings. Body temperature was maintained at 36°C.

Postmortem Evaluation

After removing the right ventricle, the excised hearts were sliced transversely into 5 sections from apex to base, which were weighed and photographed to identify the nonperfused region or area at risk (AR) (nonblue; Fig. 2). The slices were then incubated in a 1% solution of triphenyltetrazolium chloride at 37°C for 15 minutes, kept in formalin overnight and rephotographed the following morning to demonstrate the area of necrosis (AN) (pale white or yellow; Fig. 2). Computerized planimetry of the photographs was used to determine the AR and AN, which were expressed as % of the left ventricle (LV) and used to calculate the IS, expressed as % of risk area (IS = AN/AR × 100). We also measured the LV weight.

Statistics

Data are presented as mean ± SEM and analyzed using SAS software (Version 6.04, Cary, NC). Student *t* tests were performed to compare risk area, necrosis, and IS values between mildronate and control groups. The relationship between AR and AN was analyzed using analysis of covariance (ANCOVA). The hemodynamic values recorded at different time points were analyzed using 2-way analysis of variance for repeated-measures to compare variables by group and over time. Frequency of arrhythmias was analyzed using the Fisher exact test. $P < 0.05$ was considered statistically significant.

RESULTS

Hemodynamic Measurements

Heart rate in the mildronate-treated rats was similar to the heart rate in the control rats either at baseline or during occlusion or reperfusion (Table 1). Mean arterial pressure, systolic blood pressure, and diastolic blood pressure did not show significant differences between the 2 groups at all studied time points (Table 1). In both groups, however, the 3 parameters were significantly lower during the occlusion and reperfusion phase than at baseline, as expected for an ischemia/reperfusion experimental model ($P = 0.0001$, $P = 0.010$, and $P < 0.0001$, respectively). We observed no differences in the frequency of arrhythmias between the 2 groups (arrhythmias were registered in all 14 rats in the control group, and in 13 out of 16 rats in the mildronate-treated group; P = NS).

Body Weight and LV Weight

Body weight averaged 246.7 ± 6.9 g versus 242.3 ± 4.2 g at the beginning of the study, and 263.4 ± 5.5 g versus 255.8 ± 4.7 g at the end of the study in the control versus the mildronate-treated groups respectively (P = NS). The LV weight did not differ between the control (0.518 ± 0.013 g) and the mildronate-treated rats (0.523 ± 0.018 g; P = NS).

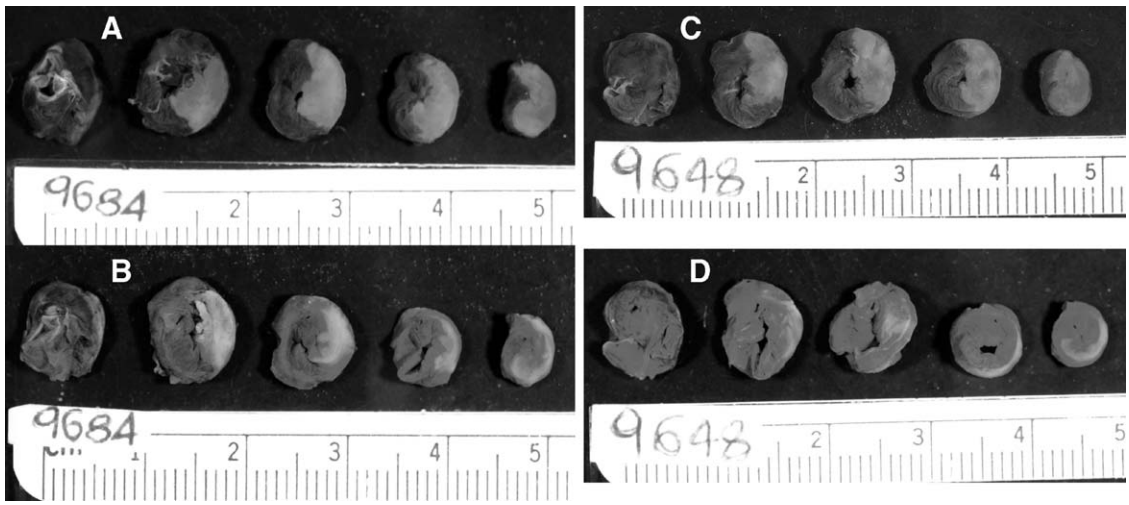

FIGURE 2. Digital pictures of a control (A and B) and a mildronate-treated (C and D) rat hearts cut transversely into 5 sections. A and C, Sections after injection of the blue dye. The blue area is the area perfused by the dye, whereas the red/pink area represents the nonperfused region or AR. B and D, Same slices after incubation in a 1% solution of triphenyltetrazolium chloride. The pale white or yellow region represents the AN, whereas the red region represents viable tissue. The risk and necrotic areas were expressed as % of the LV and used to calculate the IS, expressed as % of risk area.

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TABLE 1 Hemodynamic Variables (mean ± SEM)

	Baseline	45 min Occlusion	1 h Reperfusion	2 h Reperfusion	*P*
Heart rate (bpm)					
Control	247 ± 13	210 ± 9	204 ± 11	209 ± 8	Group NS
Mildronate	239 ± 15	216 ± 12	205 ± 14	222 ± 13	Time NS
Mean arterial pressure (mm Hg)					
Control	85 ± 5	64 ± 3	60 ± 2	63 ± 4	Group NS
Mildronate	84 ± 6	60 ± 2	63 ± 4	62 ± 2	Time 0.0001
Systolic blood pressure (mm Hg)					
Control	101 ± 5	78 ± 3	75 ± 3	78 ± 4	Group NS
Mildronate	99 ± 8	73 ± 4	77 ± 6	75 ± 3	Time 0.01
Diastolic blood pressure (mm Hg)					
Control	78 ± 5	58 ± 3	52 ± 2	55 ± 4	Group NS
Mildronate	77 ± 5	53 ± 2	57 ± 4	55 ± 2	Time < 0.0001

Statistical analysis by repeated-measures analysis of variance between groups and over the time; $P < 0.05$ statistically significant. Mildronate group, n = 16; control group, n = 14.
bpm indicates beats per minute.

IS

Planimetric analysis showed that mildronate and control groups had identical ischemic AR (expressed as a % of LV) at 38 ± 3% in the mildronate group versus 38 ± 2% in the control group (Fig. 3). However, the amount of necrosis was smaller in the mildronate group at 16 ± 2% of the LV than in the control group at 22 ± 2% ($P = 0.05$). Myocardial IS (expressed as a % of AR) was significantly reduced in the mildronate-treated rats at 43 ± 3% compared with controls at 57 ± 4% ($P = 0.004$; Fig. 3). Thus mildronate resulted in a 25% reduction in the size of the myocardial infarcts.

Relationship Between Risk and Necrotic Areas

Individual values of AR in both control and mildronate groups were analyzed and plotted against the correspondent AN values (Fig. 4). This correlation between AR and AN showed that for any amount of risk above 25% of LV, rats treated with mildronate had smaller percentage of necrosis ($P = 0.0035$, by ANCOVA).

DISCUSSION

This study for the first time demonstrated that a 10-day treatment with mildronate, a novel antianginal agent and fatty acid oxidation inhibitor, reduced myocardial IS in an experimental model of acute myocardial ischemia without affecting blood pressure or heart rate. The weight of the LV remained unchanged as well. The lack of hemodynamic effects indicates that from the pharmacological standpoint mildronate is a safe drug. Previous studies in rats demonstrated that mildronate given at a broad range of doses (ie, 100 to 800 mg/kg orally over 10 to 20 d) did not affect heart weight and caused no signs of cardiac hypertrophy or decompensation of cardiac function.[12,13]

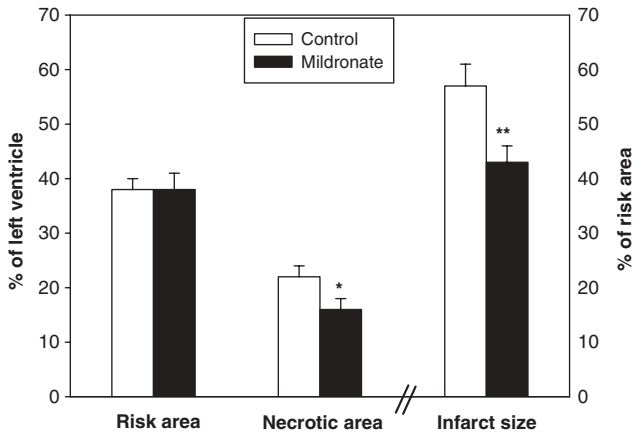

FIGURE 3. Values of risk and necrotic areas (% of the LV) in the control (open bar) and mildronate group (black bar) are reported on the left *y*-axis. On the right *y*-axis, the IS values (% of the risk area) for the control (open bar) and mildronate group (black bar) are reported. Mildronate versus control group by student *t* test; **P* = 0.05, ***P* = 0.004. Mildronate group, n = 16, and control group, n = 14.

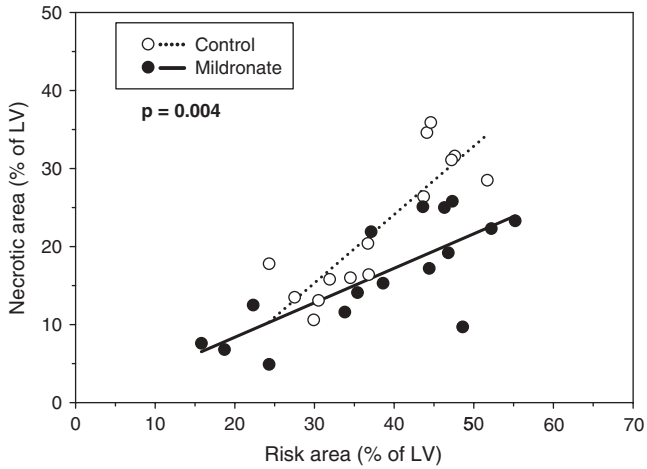

FIGURE 4. Correlation between AR and AN. For any risk area > 25%, the percent of necrosis was significantly lower in the mildronate (black symbol) than in the control group (open symbol). Statistical ANCOVA.

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Heart attack or acute myocardial infarction remains a leading cause of morbidity and mortality worldwide. Many drugs have been tested for their ability to reduce myocardial IS when administered as an adjunct to reperfusion therapy. Many agents that initially seemed promising failed to reduce the size of the myocardial infarct or improve clinical outcome in clinical trials. Examples include glucose-insulin-potassium, neutrophil inhibitors (Hu23F2G and rhuMAb CD18), complement inhibitor pexelizumab, and Na/H exchange inhibitor eniporide.[14–19] Trimetazidine, designed to balance glucose and fatty acid utilization in the ischemic heart demonstrated strong antianginal activity, but failed to reduce both IS and short-term mortality in patients with acute myocardial infarction.[20] Inhibitors of cholesterol biosynthesis, statins, although decreasing IS in experimental settings and reducing the incidence of major coronary events in patients with myocardial infarction, have never been studied clinically as IS limiting agents.[21–23] Thus, there remains a need for new therapies and new cardioprotective agents that could effectively reduce the extent of necrosis and result in a better clinical outcome.

Mildronate may be promising in this regard. Mildronate has been shown to reduce fatty acid β-oxidation by inhibiting the synthesis of carnitine[4] (Fig. 1). The decrease in carnitine levels significantly reduced the accumulation of the long-chain acylcarnitine in the cytoplasm and long-chain acyl-coenzyme A (acyl-CoA) in the mitochondria, thereby inhibiting β-oxidation of fatty acids.[4,5,10] The accumulation of the fatty acid intermediate products is cytotoxic, because these amphiphilic molecules inhibit several membrane-bound enzymes involved in the regulation of cell function.[24–30] Long-chain acylcarnitine causes cellular uncoupling in the heart, induces calcium release from the sarcoplasmic reticulum, and is considered a strong arrhythmogenic factor.[24–26] Long-chain acyl-CoA is known as an inhibitor of adenine nucleotide translocase, and its accumulation is linked to a decrease in adenosine triphosphate stores in the cytosolic compartment.[27–30] Mildronate demonstrated cardioprotection by reducing the accumulation of the fatty acid metabolites in various animal models during different conditions including hypoxia, ischemia, and congestive heart failure.[5–9,13] To achieve these effects, the administration of a course of mildronate over several days is needed.

In addition to inhibiting oxidation of fatty acid, mildronate was also shown to increase glucose oxidation in hypoxic rat hearts.[31] This effect could be explained by the close relationship between the glucose and fatty acid metabolism. Increased oxidation of fatty acid has an inhibitory effect on glycolysis and glucose oxidation, whereas stimulation of glucose metabolism inhibits oxidation of fatty acid. Inhibition of fatty acid oxidation with mildronate frees significant amounts of nicotinamide-adenine dinucleotide, which can be used in reactions catalyzed by glyceraldehyde-3-phosphate dehydrogenase and pyruvate dehydrogenase. Increased activities of both enzymes promote both glycolysis and glucose oxidation. In addition, inhibition of fatty acid oxidation with mildronate lowers the acetyl-CoA/CoA ratio in the mitochondria.[5] Decreased level of acetyl-CoA removes allosteric inhibition of pyruvate dehydrogenase, and thus stimulates oxidative decarboxylation of pyruvate, which is a key enzyme in glucose oxidation.

Among the recently discovered biochemical effects of mildronate is its ability to inhibit carnitine reabsorption in kidneys, an acute mechanism that might also contribute to the decreased concentration of free carnitine in the cardiac muscle and inhibition of fatty acid oxidation.[32] In rats with myocardial infarction, mildronate was shown to attenuate the decreases in SERCA2 protein and hexokinase I content in the ischemic heart.[33] Maintaining adequate levels of SERCA2 might be important in preserving calcium metabolism and cardiac contractility in the ischemic heart, whereas conserved levels of hexokinase I could play a significant role in the increased metabolism of glucose. In addition to the inhibition of GBBH, all the above-mentioned effects of mildronate could also be characterized as cardioprotective. Because mildronate inhibits the carnitine-dependent oxidation of long-chain fatty acid and, at the same time, promotes glucose oxidation, the energy-generating pathways are balanced and the functional state of mitochondria isolated from mildronate treated rat hearts is not affected.[34]

Mildronate is currently approved for the use of angina pectoris in Eastern Europe, after the success of several clinical investigations held in Latvia and Russia.[35–37] Despite the data on the effects of mildronate on cardiac hemodynamic dysfunction, no studies have documented the effects of a course of treatment with mildronate on IS during acute myocardial ischemia. There are 2 studies showing that 1 single dose of mildronate, when given 1 hour before or 1 hour after the ligation of the coronary artery, decreased the IS in rats,[38,39] but the effect of a course of mildronate administration on IS (as might occur in a patient receiving mildronate for angina) has never been addressed. In addition, the effect of a single mildronate dose is most probably not linked to its ability to inhibit GBBH and to decrease carnitine biosynthesis, but rather could be explained by its acute biochemical effects; for example, interference with renal reabsorption of carnitine.

After a myocardial infarction, some heart cells in the ischemic risk area are lost and usually replaced by fibrotic tissue that does not contract. This contributes to the thinning and expansion of the infarct region, which progresses with time, resulting in cardiac enlargement and cardiac failure.[40] Because cardiac function can be improved by reducing myocardial scar, it was therefore important to determine whether mildronate would have been able to reduce cardiac cell necrosis after an acute myocardial infarction. In this study, we showed for the first time that myocardial IS was reduced by 25% after a 10-day course of mildronate and that, for any amount of risk, necrosis was smaller in the mildronate group. We also showed that mildronate's effect was independent of

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any effect on hemodynamics. Mildronate did not induce any change in blood pressure, heart rate, or frequency of arrhythmias during ischemia and reperfusion.

CONCLUSION

Our findings substantiate that mildronate, an agent used for the treatment of angina pectoris, was also able to significantly reduce ischemic cell death in the setting of acute myocardial infarction without unsafe hemodynamic effects.

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