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A.V. DURKINA[1], O.G. BERNIKOVA[1], N.J. MIKHALEVA[1], N.M. PADERIN[2], K.A. SEDOVA[3], M.A. GONOTKOV[1], V.S. KUZMIN[4], J.E. AZAROV[1]

MELATONIN PRETREATMENT DOES NOT MODIFY EXTRASYSTOLIC BURDEN IN THE RAT ISCHEMIA-REPERFUSION MODEL

[1]Department of Cardiac Physiology, Institute of Physiology, Komi Science Center, Ural Branch, Russian Academy of Sciences, Syktyvkar, Russia; [2]Department of Molecular Immunology and Biotechnology, Institute of Physiology, Komi Science Center, Ural Branch, Russian Academy of Sciences, Syktyvkar, Russia; [3]Department of Biomedical Technology, Faculty of Biomedical Engineering, Czech Technical University in Prague, Kladno, Czech Republic; [4]Department of Human and Animal Physiology, Biological Faculty, Lomonosov Moscow State University, Moscow, Russia

The mechanism of reentrant ventricular tachyarrhythmias complicating acute myocardial ischemia is largely based on the interaction between an arrhythmogenic substrate and triggers. Melatonin was proposed as an antiarrhythmic medication and was shown to ameliorate the arrhythmogenic substrate. Also, melatonin provides a sympatholytic effect in different settings and might attenuate ectopic activity, which provides reentry triggers. In the present study, we aimed at evaluating the melatonin effects on cardiac sympathetic activity and the incidence of premature ventricular beats during the episode of ischemia-reperfusion. Experiments were done in a total of 26 control and 28 melatonin-treated (10 mg/kg, daily, for 7 days) male rats. Sympathetic fibers density was assessed by glyoxylic acid-induced fluorescence. Continuous electrocardiograms recording was performed during ischemia-reperfusion episodes (5 min/5 min, respectively) induced by reversible coronary occlusion. Myocardial expression of tyrosine hydroxylase, a rate-limiting enzyme of catecholamine biosynthesis was assessed by Western blotting. No differences in the state of sympathetic innervation were observed in histochemical analysis. However, Western blotting analysis demonstrated that melatonin treatment suppressed tyrosine hydroxylase expression in the non-ischemic ($p < 0.05$ versus control) but not ischemic regions of myocardium. The melatonin-treated animals had longer RR-intervals in the baseline state than the control animals (264 ± 48 ms versus 237 ± 33 ms, $p = 0.044$, respectively), but this difference decayed during the period of ischemia due to the increase of heart rate in the treated group. The number of premature ventricular beats did not differ between the control and treated groups during the ischemic and reperfusion periods. One-week melatonin pretreatment caused a slight peripheral sympatholytic effect that attenuated during ischemia and completely disappeared by the onset of reperfusion. The slight expression of sympathetic downregulation was associated with the lack of any effect of melatonin on extrasystolic burden. Collectively, the data suggest that melatonin cannot target the triggers of reentrant arrhythmias.

Key words: *ventricular tachycardia, ventricular fibrillation, melatonin, ischemia-reperfussion, sympathetic activity, heart rate, extrasystoles, tyrosine hydroxylase, premature ventricular beats*

INTRODUCTION

Ventricular tachycardia and/or ventricular fibrillation (VT/VF) are a frequent and life-threatening complication of myocardial ischemia, and prevention of such arrhythmic events presents an important challenge. VT/VFs largely result from reentrant mechanisms based on interaction of an arrhythmic substrate and trigger (1-3). The latter is provided by ventricular extrasystoles with short coupling intervals usually caused by early or delayed afterdepolarizations or by abnormal automaticity. Development of safe and effective antiarrhythmic drugs presents a significant research challenge. Potential cardioprotective properties of many natural substances such as coenzyme Q10 (4), vitamin D (5), and plant extracts (6) have been studied. Melatonin, a secretory product of the pineal gland has been also suggested as a promising cardioprotective and antiarrhythmic agent.

Melatonin demonstrated antiarrhythmic properties in ischemia/reperfusion conditions (7-9). It consistently prevented reperfusion VT/VF in rodent *in vivo* or isolated heart preparations. However, in a setting of epinephrine-induced myocardial injury melatonin did not cause statistically significant effects on arrhythmias (10). This inconsistency raises questions about the mechanisms of its action. Melatonin promoted post-ischemic recovery of action potential duration (8-11) and activation time (11). Both of these effects can be interpreted as amelioration of the reentry substrate. However, little is known whether melatonin modifies the triggers of reentrant arrhythmias, i.e. the extrasystolic burden.

Though melatonin can act as an antioxidant agent (12), our previous study has demonstrated that the antiarrhythmic effect of melatonin was independent of its antioxidative properties (11, 13). Another mechanism, which might be involved in realization

of the electrophysiological effects of melatonin, specifically in regard to extrasystolic burden is its prevention of sympathetic overactivation, which is recognized to be proarrhythmic (14). Melatonin modifies peripheral sympathetic activity *via* central mechanisms (15). Recent data suggest that melatonin lowers the level of norepinephrine in the heart (16). Among other mechanisms, the suppression of tyrosine hydroxylase activity may account for this effect in the model of ischemia/reperfusion (17). However, in other experimental models melatonin increases tyrosine hydroxylase activity thereby enhancing synthesis of catecholamines (18-20). Since sympathetic activity promotes ectopic activity by facilitation of both abnormal automaticity and afterdepolarizations (21-24) and predisposes to ischemia-induced VF (25), melatonin-related changes of extrasystolic burden might be expected.

In the present work, we addressed a question whether melatonin modifies the arrhythmic triggers and sympathetic activity. The aim of the study was evaluation of the effects of a week-long melatonin treatment on catecholamine production in the heart, heart rate as a functional manifestation of cardiac sympathetic activity, and the number of premature ventricular beats (PVB) as a quantitative measure of extrasystolic burden serving as reentry trigger.

MATERIALS AND METHODS

Animals

Experiments were performed in a total of 54 Wistar male rats (3-month-old, body mass 150 – 50 g) from the Collection of Experimental Animals of the Institute of Biology, Federal Research Center, Komi Scientific Center, Ural Branch of the Russian Academy of Sciences.

The study conformed to the Guide for the Care and Use of Laboratory Animals, 8th Edition published by the National Academies Press (US), 2011, the guidelines from Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes, and was approved by the ethical committee of the Institute of Physiology of the Komi Science Centre, Ural Branch of Russian Academy of Sciences (approval 19 February 2018, amendment 19 September 2018).

Before entry in the study, the animals were quarantined for one week. The animals were kept in standard laboratory cages under the standard condition (12 hours light/dark cycle). For seven days, the animals daily received single oral doses of melatonin (10 mg/kg, melatonin group) or placebo (control group) always at the same time of 4 pm. Melatonin or placebo was given before feeding in small pellets (1/2 teaspoon of peanut butter mixed with or without melatonin powder, respectively). The animals were closely observed to have eaten the pellet completely before regular food was given. For sake of consistency, the dosage and duration of the treatment was the same as in our previous work (11). The apparently high dose of 10 mg/kg was due to approximately 50% bioavailability of oral melatonin (26).

Fig. 1 illustrates a general design of the study. It included electrophysiological experiments, tyrosine hydroxylase measurements and catecholamine histochemistry. In line with 3R principles we did our best to provide an optimal combination of measurements applied in the same animals (refine) and reduced the number of animal experiments (replacement was not appropriate for this work). To do this, we extracted information on heart rate and PVB from the data obtained in 10 melatonin-treated and 8 control rats from our study with the same experimental protocol (11) (the data used in the present study have not been previously reported).

Ischemia-reperfusion model

Twenty two animals of the melatonin group and 20 animals of the control group were anesthetized with zoletil (Virbac S.A., Carros, France, 15 mg/kg, i.m.) and xylazine (Interchemie, Castenray, Netherlands, 0.1 mg/kg, i.m.) and ventilated mechanically. The heart was exposed by a midsternal incision and kept warm (37 – 38°C). A ligature was tied around a proximal third of the left anterior descending coronary artery (LAD) by a coated braided polyester ligature (No 5-0, Ti-Cron, Cardiopoint, Covidien, Dominican Republic). LAD occlusion was kept for 5 minutes, then the ligature was loosened to initiate reperfusion. After 5 minutes of reperfusion the animals were euthanized by potassium chloride infusion, and the hearts were quickly excised for postmortem analysis. All experiments were performed in the morning hours from 8 am to noon.

Heart rate and ectopic activity assessment

Standard bipolar limb lead electrocardiograms (ECGs) were continuously recorded from anesthesia induction until the end of experiment. Heart rate (expressed as RR intervals) was assessed at baseline, at the 1st, 2nd, 3rd, 4th, and 5th minutes of coronary occlusion and at the 1st, 2nd, 3rd, 4th, and 5th minutes of reperfusion. Extrasystolic burden was characterized by the number of the PVB, which was measured at the same time-points as RR intervals excluding the baseline since PVB were quite rare before coronary occlusion and were due to surgical manipulations.

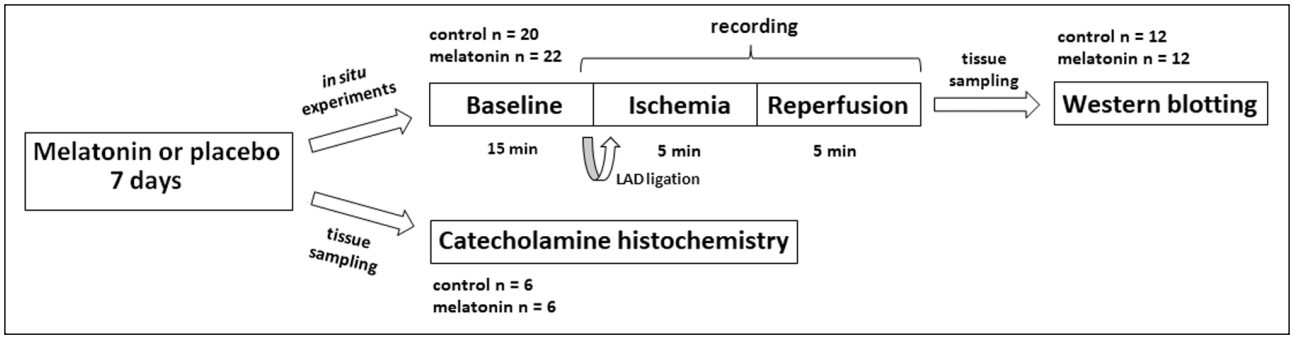

Fig. 1. Experimental design for the study.
LAD, left anterior descending coronary artery.

Western blotting

Western blotting was performed in the heart preparations taken from non-ischemic (apex of the right ventricle) and ischemic regions (apex of the left ventricle) of 12 melatonin-treated and 12 control animals undergone ischemia-reperfusion. The tissue samples were homogenized for 5 min at 4°C in 0.2 ml of lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 50 mM NaF, 5 mM EDTA, 0.1% SDS, 0.1% Triton X-100, 1 mM PMSF, 1 mM Na_3VO_4 and protease inhibitor cocktail (Sigma-Aldrich, St, Louis, MO, USA) at a 1:100 dilution), centrifuged at 4°C at 10,000 g for 10 min and then stored at –40°C until measurements.

The concentration of the protein extracts was determined with a bicinchoninic acid protein assay kit (Pierce Biotechnology/ThermoFisher Scientific, Waltham, MA, USA). For each protein sample, 10 µg was diluted in 1 × SDS loading buffer (3.55 ml of ddH_2O; 1.25 ml of 0.5M Tris-HCl, pH 6.8; 2.5 ml of glycerol; 2.0 ml of 10% (w/v) SDS; and 0.2 ml of 0.5% (w/v) bromophenol blue), boiled for 5 min and loaded on to a 10% SDS-polyacrylamide denaturing gel. Then, the electrophoresis gels were blotted onto nitrocellulose membranes by electrophoretic transfer. The filters were then incubated with the appropriate antibody (anti-TH, cat #MA1100, anti-actin, cat #MA1000, and HRP-conjugated goat anti-mouse IgG, cat #BA1050, all Boster Biological Technology, Pleasanton, CA, USA), and the immunoreactive bands were detected by using the Immune-STAR HRP substrate Kit (Bio-Rad Labs. Inc., Des Plaines, IL, USA) according to the manufacturer's instructions. The light emission was captured with the ChemiDoc MP Imaging System (Bio-Rad, Labs. Inc., Des Plaines, IL, USA).

Assessment of sympathetic fibers density by catecholamine histochemistry

The hearts of the 6 control and 6 melatonin-treated rats not subject to ischemia/reperfusion insult were rapidly excised under deep anesthesia, washed through the aorta and coronary system with Tyrode's solution (NaCl 150 mM (Vecton, Russia); KCl 5.4 mM (Acros Organics, Geel, Belgium); $MgCl_2$ 1.2 mM (Sigma-Aldrich, St. Louis, MO, USA); $CaCl_2$ 1.8 mM (Sigma-Aldrich, St. Louis, MO, USA); HEPES sodium salt 5 mM (Acros Organics, China); D-glucose 5.6 mM (Sigma-Aldrich, St. Louis, MO, USA); pH 7,4). The left ventricle free wall (LV) 20-30 µm transmural cross-sections and were dissected immediately after the washout. The samples were taken from the same regions halfway between the base and the apex in all hearts.

The preparations were incubated for 30 minutes in a modified phosphate buffered saline (PBS) solution (NaCl 0.09 M; Na_2HPO_4 0.01 M (Acros Organics, Geel, Belgium)) containing 2% glyoxylic acid monohydrate (Sigma-Aldrich, St. Louis, MO, USA) and 10% sucrose (Sigma-Aldrich, St. Louis, MO, USA), the pH of which was adjusted to 7.4 by 1 M NaOH. (Sigma-Aldrich, St. Louis, MO, USA). After the incubation the preparations were mounted on objective glasses and desiccated in 45°C airflow during 30 min in addition to 5 min exposure in dry air thermostat at 100°C. The dried preparations were poured with liquid paraffin and covered with a coverslip.

In order to standardize the fluorescence outcome, imaging of the specimens was carried out on the next day after preparation under same room temperature and humidity. The catecholamine-derived fluorescence was analyzed in the midmyocardial area 200 – 400 µm distantly from endo- and epicardial surfaces to exclude inhomogeneity of catecholamine-positive fibers distribution. Since the wall of intramural coronary arteries has abundant sympathetic innervation, the perivascular regions were excluded from the areas of interest with aim to eliminate contribution of "nonmyocardial" fibers from the analysis.

Confocal microscope Zeiss LSM700 with air Plan-Apochromat 20x/0.8 M27 objective was used to visualize glyoxylic acid-induced fluorescence in the immersed LV preparations. The emitted fluorescence was detected in confocal mode with 0.56 µm pinhole in 405–480 nm (maximum at 435 nm) wavelengths range and was induced by diode excitation 405 nm laser. Confocal 2048x2048 px images included 25 stacks, which covered the entire tissue samples thickness were recorded using Carl Zeiss ZEN 7.0 software.

Collected data were analyzed off-line by ImageJ 1.50i software. Open-source Bio-Formats Explorer ImageJ plugins (imagej.net/Bio-Formats) were used to handle images. After background subtraction and binarization, a total amount of fluorescence-positive pixels as well as total length of the fibers were calculated as an indicator of the density of catecholamine-positive fibers in the tissue. All tissue preparations were stained, scanned, processed and quantified using the same protocol.

Statistical analysis

Statistical analysis was performed with the SPSS package (IBM SPSS Statistics 23). According to the Kolmogorov-Smirnov test, the parametric Student t-test and nonparametric Mann-Whitney test were used for melatonin versus control comparisons of the electrophysiological data (RR intervals and PVB) and the data on the sympathetic system properties (tyrosine hydroxylase expression and sympathetic fiber density), respectively. For repeated measurements in the same animals, Friedman and Wilcoxon tests were used for multiple and paired comparisons, respectively. Association between the number of PVB and heart rate was assessed by linear regression analysis. The diferences were considered significant at $p < 0.05$.

RESULTS

Heart rate and extrasystolic activity

In baseline, all the animals demonstrated sinus rhythm (*Fig. 2A*). PVB developed during occlusion and reperfusion, especially at the first minutes of both phases. *Fig. 2B* displays dynamics of heart rate expressed as RR interval duration during ischemia and reperfusion phases in the treated and control animals. Baseline RR intervals were longer in the melatonin group as compared to the controls (mean ± SD: 264 ± 48 ms versus 237 ± 33 ms, $p = 0.044$, respectively). However, this difference decayed during the period of coronary occlusion as heart rate progressively increased in the melatonin group attaining the level of the controls by the end of 5-min ischemia period. During reperfusion, no significant differences between the groups and no significant temporal evolution of RR intervals within both groups were observed.

PVBs developed largely at the onset of ischemia and during reperfusion (*Fig. 2C*). In linear regression analysis, the PVB incidence was associated with RR interval duration during reperfusion but not during ischemia (regression coefficient –0.039, 95% CI –0.074 – –0.004, $p = 0.027$, and regression coefficient –0.018, 95% CI –0.044 – 0.008, $p = 0.170$, respectively). The PVB number did not differ between the groups during both ischemia and reperfusion periods.

Tyrosine hydroxylase expression and sympathetic fiber density

Western blotting (*Fig. 3*) showed that melatonin treatment was associated with a decreased level of tyrosine hydroxylase

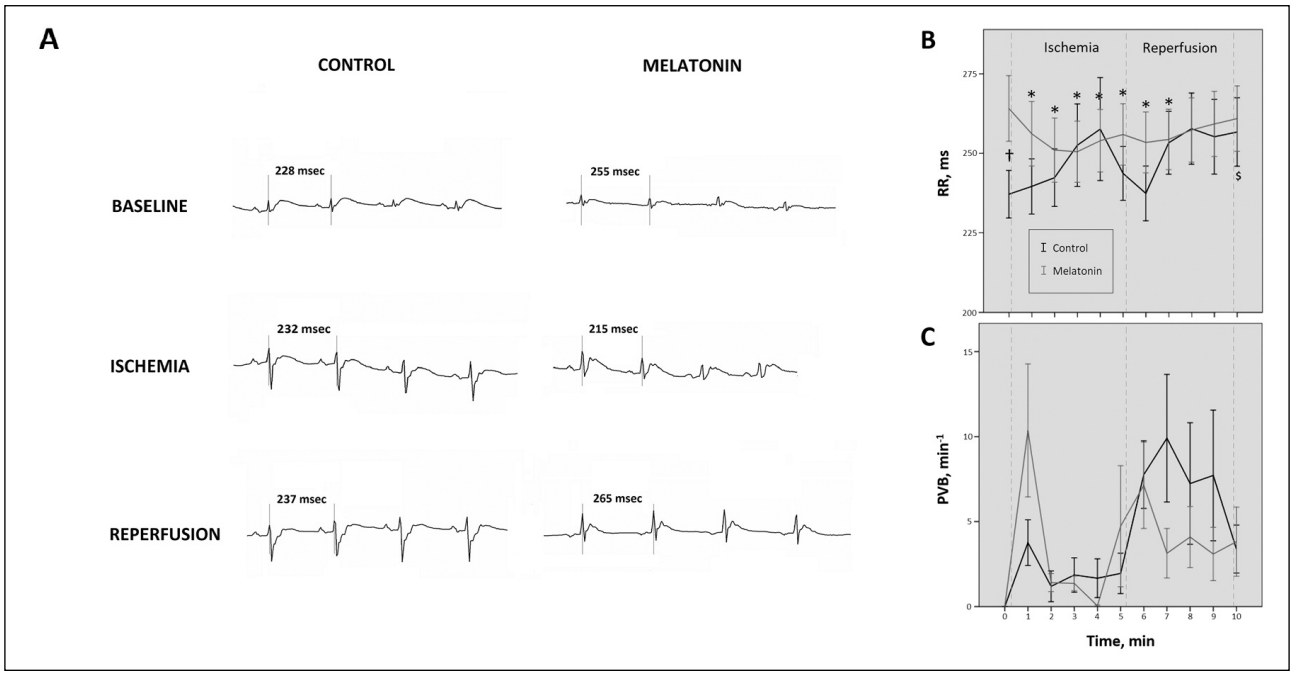

Fig. 2. Dynamics of electrophysiological variables during ischemia-reperfusion episodes. (*A*) displays representative limb lead II electrocardiograms of the treated and control animals in baseline, ischemia and reperfusion. (*B*) and (*C*) show evolution of RR-interval (*B*) and the number of premature ventricular beats (PVB, (*C*)) during coronary occlusion and reperfusion in melatonin-treated and control rats (mean ± SEM). * $p < 0.05$ versus baseline in the melatonin group, $^{\$}$ $p < 0.05$ versus baseline in the control group, † $p = 0.041$ melatonin (n = 22) versus control (n = 20).

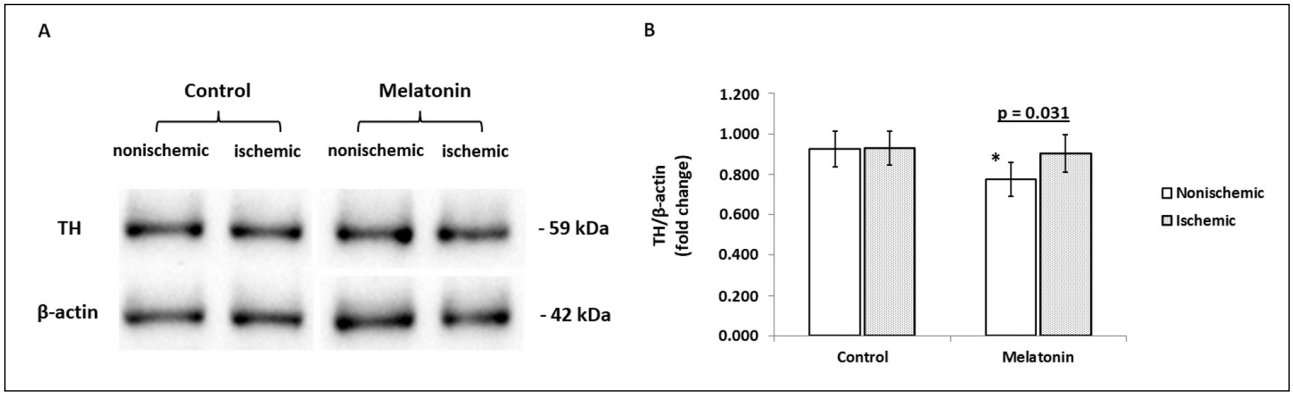

Fig. 3. Tyrosine hydroxylase (TH) protein levels in the ischemic and nonischemic zones in the control (n = 12) and melatonin (n = 12) groups. (*A*): Representative bands of tyrosine hydroxylase and β-actin. (*B*): Bar graph showing TH/β-actin as fold change (mean ± SEM). * $p < 0.05$ compared with control.

expression in the non-ischemic zone as compared to the control group. As a result, a significant difference in tyrosine hydroxylase expression was found between the non-ischemic and ischemic zones in the treated animals. No differences in tyrosine hydroxylase expression were found between the non-ischemic and ischemic zones in the control animals. Also, tyrosine hydroxylase expression in the ischemic zone did not differ in the control and melatonin group.

Sympathetic fiber density was evaluated in the ventricular samples taken from the hearts without induction of ischemia as the fluorescence of catecholamine adducts with glyoxylic acid. No differences in the sympathetic fiber density were found between the control and melatonin groups (*Fig. 4*).

DISCUSSION

In the present investigation, we found that 7-day melatonin treatment did not modify the density of sympathetic nerve fibers but decreased expression of tyrosine hydroxylase in the non-ischemic myocardial tissue. Also we found a decrease in the baseline heart rate in the melatonin group. However, the extrasystolic burden did not differ between the control and melatonin-treated animals.

Melatonin might be related to reentrant arrhythmogenesis in several ways including direct cardiac electrophysiological, antioxidative and sympatholytic effects (*Fig. 5*). Previously, the antiarrhythmic effect has been reported to be independent of

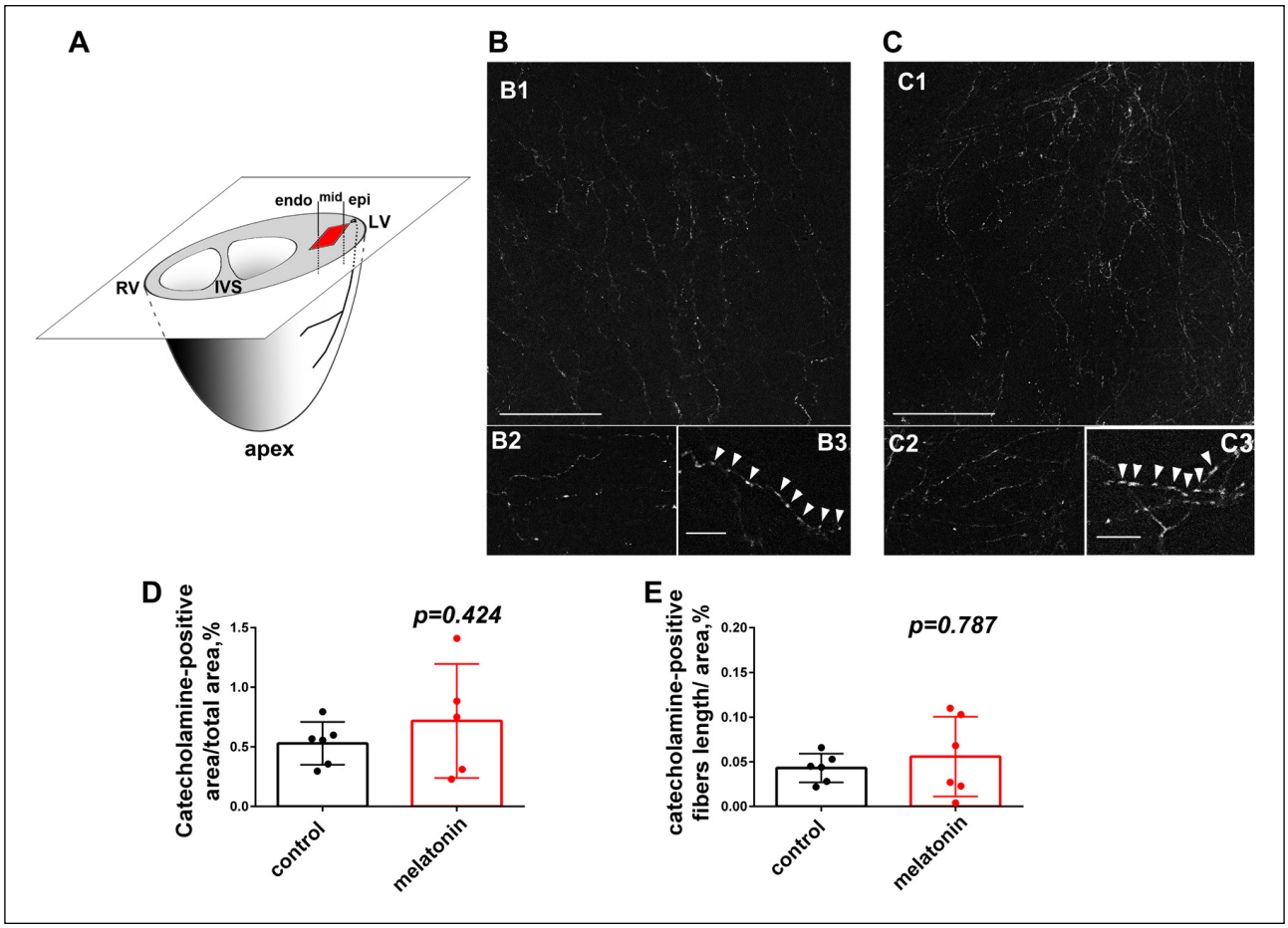

Fig. 4. Catecholamine-positive fibers in the ventricular myocardium of control and melatonin-treated rats. (*A*): Schematic representation showing sites in the left ventricle where sympathetic fibers were estimated. (*B*), (*C*): Representative examples of the confocal images of various magnification (*B1-B3, C1-C3*) showing catecholamine-positive fibers in the left ventricle from control (*B*) and melatonin-treated rats (*C*). Arrowheads (*B3* and *C3*) show spots with high intensity of the fluorescence which can be associated with sympathetic nerve varicoses. Scalebars - 100 (*B1, C1*) and 10 (*B3, C3*) μm. (*D*), (*E*): An area of the catecholamine-derived fluorescence (*D*) and a length of the catecholamine-positive fibers (*E*) normalized for the total area of the samples from control and melatonin-treated rats.

Abbreviations: RV, LV, right and left ventricular wall, respectively; IVS, interventricular septum; endo-, mid-, epi- endocardium, midmyocardium, epicardium, respectively; rectangle indicates an analyzed area in the midmyocardium in the left ventricle.

melatonin antioxidative properties but related to direct amelioration of the arrhythmogenic substrate, specifically enhancement of ventricular activation (11, 13). The present investigation sought to explore the influence of melatonin on the second (other than the substrate) prerequisite of reentry arrhythmogenesis, namely trigger mechanisms, which at least in part might be related to a probable sympatholytic effect. Such an influence was expected since there is evidence on sympatholytic effect of acute and chronic melatonin treatment (15-17, 27-29), and a relation between sympathetic activity and abnormal automaticity and/or afterdepolarization is well documented (21-24). For the evaluation of density and functional activity of the sympathetic fibers, we used the glyoxylic acid derived fluorescence method and Western blotting of tyrosine hydroxylase in ventricular samples. Tyrosine hydroxylase is a rate-limiting enzyme of catecholamine biosynthesis. It uses tetrahydrobiopterin and molecular oxygen to convert tyrosine to L-DOPA. The products of this pathway (dopamine, epinephrine and norepinephrine) are released in the central and peripheral nervous systems as well as in the medulla of adrenal glands and act as hormones and neurotransmitters (for review see (30)). The assessment of glyoxylic acid derived fluorescence is a standard approach used for estimation of the sympathetic innervation in the heart as well as for estimation of catecholamines content in the tissue directly showing all fractions of catecholamines. For our purpose, the glyoxylic acid fluorescence method has an advantage over an immunohistochemical identification of the sympathetic fibers since the latter would not correlate with a functional state of the fibers (catecholamines content).

We found that although melatonin treatment did not modify sympathetic fibers network, it lowered the level of tyrosine hydroxylase expression in the non-ischemic myocardium. This effect corresponded to the melatonin-associated decrease in heart rate in the non-ischemic state reported previously (31). However, this action of melatonin had significant limitations. Firstly, tyrosine hydroxylase downregulation was observed only in the non-ischemic region of the ventricular myocardium. Secondly, the initially decreased heart rate in the treated animals rapidly increased during the episode of ischemia-reperfusion and attained the level of untreated animals. A probable explanation

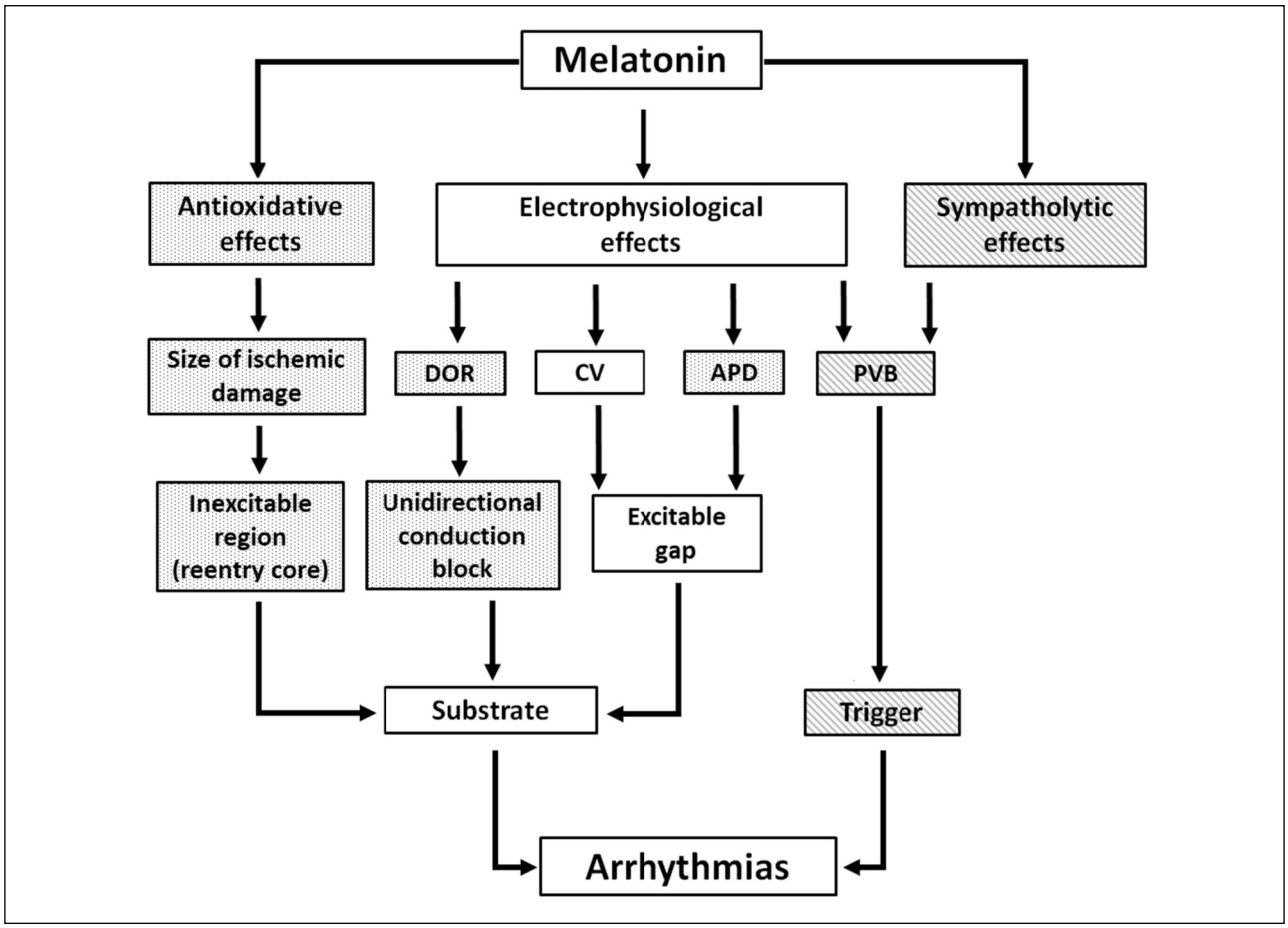

Fig. 5. Schematic representation of melatonin action on electrophysiological targets significant for reentry development. Reentry arrhythmias require for their development the simultaneous presence of a trigger and vulnerable substrate. The trigger is provided by a premature ventricular beat (PVB), while the substrate has three essential components, namely an inexcitable region, unidirectional conduction block and excitable gap. The region of inexcitability forms due to ischemic damage. The unidirectional conduction block develops in conditions with increased dispersion of repolarization (DOR). The excitable gap can be estimated as a ratio of the tissue volume to the excited tissue volume with the latter depending on the product of conduction velocity (CV) and action potential duration (APD). All the listed electrophysiological targets might be affected by melatonin via direct influences on myocardial electrophysiological properties, antioxidative effects or sympatholytic effects. Shading indicates effectiveness of individual targets and pathways in relation to antiarrhythmic action of melatonin. The pathway tested in the present study and excluded as an antiarrhythmic mechanism is shown in dashed blocks. Open and dotted blocks indicate effective and ineffective targets, respectively, according to our previous study (11).

for these effects is based on the fact that ischemia is known to cause sympathetic overactivation (1). Specifically, hypoxia stimulation can increase the level of tyrosine hydroxylase expression as well as its functional activity (30). Such sympathetic overactivation during ischemia counteracted the preischemic downregulation of sympathetic activity in the melatonin group. Collectively, our observations suggest that ischemia-induced sympathetic overactivation was sufficient to overcome the possible sympatholytic effect of melatonin.

Since sympathetic activity is related to heart rate and ectopic activity in ischemic conditions, we expected to find an association between heart rate and extrasystolic burden. Though during coronary occlusion no association was observed, the number of PVB and RR intervals were inversely associated with each other during reperfusion. At this period, the effect of melatonin treatment on heart rate was no longer observed. It is also noteworthy that PVB occurrence is not necessarily related to sympathetic activation but could be due to direct ischemic modification of connexin properties (32), generation of early (33) or delayed (34) afterdepolarizations. Specifically, changes of action potential duration in the border zone can facilitate afterdepolarizations and ventricular fibrillation (35). Whatever the exact mechanism of PVB, melatonin did not modify the extrasystolic burden in the experimental model used in the present study.

Limitations of the study concern several points. Ischemia-reperfusion was induced in otherwise healthy animals that differs from a clinical setting. Duration of ischemia episode was quite short and insufficient for infarct development. It is probable that the increase of ischemia duration would have resulted in generation of ischemic (not only reperfusion) PVB that might have demonstrated other dynamics and other sensitivity to melatonin treatment. Among hemodynamical parameters only heart rate was studied that expressed the level of sympathetic tone indirectly. It is not excluded that the reported effects might be due to changes in arterial blood pressure that

was not measured in the experiments. Though the decrease in the expression level of tyrosine hydroxylase was demonstrated, a quantitative assessment of norepinephrine level was not performed in this work and the only functional correlate of different protein expression levels was the difference in heart rate. However, PVBs were the end-points of the present study and whatever the mechanisms of their development, the absence of melatonin effect on PVB incidence was documented.

The present study demonstrated the sympatholytic action of melatonin treatment manifesting as downregulation of cardiac tyrosine hydroxylase expression and decreased heart rate. However, this effect decays during the period of ischemia. The extrasystolic burden was not subject to melatonin probably due to its insufficient sympatholytic action or other factors involved in PVB origin. Since in the context of life-threatening reentrant arrhythmias the ectopic activity serves as the triggering mechanism, the absence of the effect of melatonin on PVB development could limit its antiarrhythmic action only to modification of the vulnerable arrhythmogenic substrate.

Authors' contribution: A.V. Durkina, O.G. Bernikova, J.E. Azarov - concept and design; A.V. Durkina, O.G. Bernikova, N.J. Mikhaleva, N.M. Paderin, K.A. Sedova, M.A. Gonotkov, V.S. Kuzmin - experiments and data processing; A.V. Durkina, O.G. Bernikova, J.E. Azarov - analysis; A.V. Durkina, J.E. Azarov - drafting the manuscript. All authors read, revised, and approved the final manuscript.

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Conflict of interests: None declared.

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Author's address: Aleksandra V. Durkina, Department of Cardiac Physiology, Institute of Physiology, Komi Science Center, Ural Branch, Russian Academy of Sciences, 50, Pervomayskaya st., Syktyvkar, 167982, Russia.
E-mail: sashadurkina@mail.ru