The Effect of Continuous Light Exposure of Rats on Cardiac Response to Ischemia-Reperfusion and NO-Synthase Activity

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Running title: Continuous light modulates cardiac I/R response and NOS

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Summary

Factors modulating cardiac susceptibility to ischemia-reperfusion (I/R) are uninterruptedly attracting attention of experimental cardiology. We investigated, whether continuous 24 h/day light exposure of rats can modify cardiac response to I/R, NO-synthase (NOS) activity and the level of oxidative load represented by conjugated dienes (CD) concentration. Two groups of male adult Wistar rats were studied: controls exposed to normal light/dark cycle (12 h/day light, 12 h/day dark) and rats exposed to continuous light for 4 weeks. Perfused isolated hearts (Langendorff technique) were exposed to 25 min global ischemia and subsequent 30 min reperfusion. The recovery of functional parameters (coronary flow, left ventricular developed pressure, and contractility and relaxation index) during reperfusion as well as the incidence, severity and duration of arrhythmias during first 10 min of reperfusion were determined. The hearts from rats exposed to continuous light showed more rapid recovery of functional parameters but higher incidence, duration and severity of reperfusion arrhythmias compared to controls. In the left ventricle, the NOS activity was attenuated, but the CD concentration was not significantly changed. We conclude that exposure of rats to continuous light modified cardiac response to I/R. This effect could be at least partially mediated by attenuated NO production.

Key words: heart, melatonin, reperfusion injury, nitric oxide synthase, free radicals

Introduction

The ischemic heart disease is one of the leading causes of morbidity and mortality in developed countries (Leaf and Kang 1996). The pathomechanism of ischemia-reperfusion injury is involved in the development of myocardial infarction and cardiac arrhythmias that are the most serious manifestations of this disease. Therefore, mechanisms modulating myocardial response to ischemia-reperfusion remain to be a hot topic of experimental cardiology.

Several studies have demonstrated that continuous light exposure influences the cardiovascular system (Briaud *et al.* 2004) and abolishes nocturnal rise of melatonin blood levels (Brown *et al.* 1991). This ´functional pinealectomy´ represents a more physiological model of reduced melatonin levels as compared with surgical pinealectomy (removal of epiphysis), which decreases, beside the night-time melatonin level, the day-time melatonin level as well (Brown *et al.* 1991), temporarily increases blood pressure (Zanoboni and Zanoboni-Muciaccia 1967) and induces myocardial fibrosis (Mizrak *et al.* 2004). Deficient melatonin production may result in the lack of antioxidant action of melatonin (Girouard *et al.* 2004) and impairment of endothelial NO production, which was suggested to be enhanced by chronic melatonin treatment (Šimko and Paulis 2007).

In the present study, we investigated whether four-week exposure of experimental rats to 24 h/day continuous light can modify cardiac response to ischemia-reperfusion and modulate cardiac NO-synthase (NOS) activity or oxidative load, expressed by the concentration of conjugated dienes (CD).

Materials and methods

Experimental animals

The experiments were performed on adult male Wistar rats (body weight 250-320 g), in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). Rats were housed under standard laboratory conditions and fed with standard pellet diet and tap water *ad libitum*. The room for rats was windowless with automatic regulation of light/dark cycle. Controls were housed under standard (12 h/day light, 12 h/day dark) day cycle and animals exposed to continuous light under 24 h/day light (450 lux) for 4 weeks.

Perfusion of the hearts

The animals (n=11 in each group) were anaesthetized with pentobarbitone sodium (60 mg/kg b.w. i.p.) and treated with heparin (500 IU i.p.). Then the hearts were excised, immersed into physiological saline solution (4°C) and subsequently retrogradely perfused with carbogen (95% O_2, 5% CO_2) oxygenated Krebs-Henseleit solution (in mmol/l: 118.0 NaCl, 3.2 KCl, 1.2 $MgSO_4$, 25.0 $NaHCO_3$, 1.18 KH_2PO_4, 2.5 $CaCl_2$ and 11.1 glucose, pH 7.4, 37°C) at a constant perfusion pressure 75 mm Hg (Langendorff technique). A water-filled non-elastic balloon, coupled to a pressure transducer (MLP844, ADInstruments), was inserted into the left ventricular cavity *via* an incision in the left atrium. Diastolic pressure was adjusted to 5-7 mmHg. For epicardial electrogram recording and stimulation, an electrode was attached to the apex of the heart. Data were continuously recorded on PC using PowerLab Chart 5 (ADInstruments Ltd, Bella Vista, Australia).

The perfusion protocol consisted of 20 min standard perfusion (stabilization) and 25 min global normothermic ischemia followed by 30 min reperfusion. Electrical stimulation (300 bpm) was used except of the duration of ischemia and the first 10 minutes of reperfusion, when reperfusion arrhythmias were determined.

Analysis of reperfusion arrhythmias

Reperfusion arrhythmias in the first 10 minutes of reperfusion were evaluated using the Lambeth Conventions (Walker *et al.* 1988). The incidence and the summary duration of sever reperfusion arrhythmias (ventricular tachycardia, VT, ventricular fibrillation, VF and sustained ventricular fibrillation lasting more than 2 min, SVF) were determined. The arrhythmia score was evaluated on the base of the most sever arrhythmia occurred (1 – single premature ventricular complexes, 2 – salvos, 3 – VT (4 or more consecutive premature ventricular complexes), 4 – reversible VF and 5 – SVF).

Analysis of functional parameters

Coronary flow (CF), left ventricular systolic pressure (LVSP), left ventricular diastolic pressure (LVDP), +dP/dt max (contractility index), -dP/dt max (relaxation index) and left ventricular developed pressure (LVDevP, the difference between LVSP and LVDP) were determined.

Post-ischemic values of functional parameters (from the 15^{th}, 20^{th}, 25^{th} and 30^{th} min of reperfusion) were expressed as percentage of the base-line pre-ischemic values of these parametres, measured during the 30^{th} min of stabilization.

Conjugated dienes concentration

Probes form left ventricles (n=8 in each group) were homogenized in 15 mmol/l EDTA containing 4% NaCl. Lipids were extracted using 1:1 chloroform-methanol mixture. The concentration of CD was estimated as described by Kogure *et al.* (1982). Chloroform was evaporated in N_2 atmosphere. After the addition of 3 ml cyclohexane the absorbance at 233 nm (GBC UV/VIS 911 A) was determined. The amount of CD was calculated using the extinction coefficient ε=29 000 l/mol/cm and expressed as μmol per g tissue.

NO-synthase activity assay

Total NOS activity was determined in crude tissue homogenates of the left ventricles (n=6 in each group) by measuring the formation of [^{3}H]-citrulline from [^{3}H]-L-arginine (Amersham International plc, UK) (Bredt and Snyder 1990), with some modifications **(**Bernátová *et al.* 1999**)**. In short, 20% homogenates were centrifuged 20 min at 13 000 g. 50 μl of supernatant was incubated in the presence of 50 μl reaction agent (0.1 mol/l Tris-HCl, pH 7.4; 2 mmol/l Ca^{2+}; 10 μmol/l FAD:FMN, 1:1; 30 nmol/l calmoduline; 1 mmol/l β-NADPH; 500 μmol/l BH_4; 2 μmol/l L-arginine and 2 μl [^{3}H]-L-arginine (specific activity 5 GBq.mmol^{-1}, 100 000 dpm). After 30 min incubation at 37°C, the reaction was stopped by addition of 1 ml ice-cold stop agent (0.02 mol/l HEPES, pH 5.5; 2 mmol/l EDTA, 2 mmol/l EGTA, 1 mmol/l L-citruline). 1 ml of reaction products was applied to Dowex 50 WX-8 columns (Na^+ form). [^{3}H]-citruline content was measured by liquid scintillation counting (TriCarb, Packard, UK). NOS activity was expressed as picokatal per gram of protein (pkat / g protein).

Statistics

Data are presented as means ± standard error of mean (SEM). Results were considered significant if $p<0.05$. Two-tailed unpaired Student t-test for normally distributed values and Mann-Whitney u-test for values not normally distributed was used. Normality of distribution was tested according to Kolmogorov and Smirnov and the difference in standard deviations was tested by Barlett´s test.

Results

Heart weight and body weight

Heart weight of control rats was 0.88 ± 0.02 g, the exposure of rats to continuous light increased heart weight by 24 % ($p<0.05$). Body weight of control rats was 264 ± 3.5 g, the exposure of rats to continuous light increased body weight by 23 % ($p<0.05$). The relative heart weight (heart weight/body weight) was 3.34 ± 0.05 g/kg in controls and the exposure of rats to continuous light had no significant effect on the relative heart weight.

Pre-ischemic values of functional parameters

There were no significant differences between the groups in the functional cardiovascular parameters (coronary flow, left ventricular developed pressure, contractility index and relaxation index) at the end of stabilization (Table).

Recovery of functional parameters during reperfusion

During reperfusion the course of recovery of all investigated functional parameters: coronary flow (Figure 1A), left ventricular developed pressure (Figure

1B), contractility index (Figure 2A) and relaxation index (Figure 2B) was significantly improved in the hearts from rats exposed to continuous light.

Reperfusion arrhythmias

The incidence (Figure 3A) and summary duration of severe reperfusion arrhythmias (VT, VF, SVF) (Figure 3B), as well as the arrhythmia score (Figure 4) was significantly higher in the hearts from rats exposed to continuous light in comparison to hearts from control rats.

Conjugated dienes concentration

In the control group the CD concentration in the left ventricle was 1.31 ± 0.16 μmol/g of left ventricular tissue. Continuous light exposure of rats caused an increase of CD concentration by 34 % (non-significant) in the left ventricle (Figure 5A).

NO-synthase activity

In the control group the NOS activity in the left ventricle was 11.50 ± 1.44 pkat/g of protein. Continuous light exposure of rats caused decline in NOS activity in the left ventricle by 35 % ($p<0.05$) (Figure 5B).

Discussion

We investigated the effect of 4-week continuous light exposure of experimental rats on cardiac response to ischemia-reperfusion, NOS activity and CD concentration. Although the hearts from light-exposed rats showed more rapid recovery of functional parameters, the incidence, summary duration and severity of reperfusion arrhythmias in this group were higher. The cardiac NOS was attenuated, but the concentration of CD dienes was not significantly altered.

The surgical removal of epiphysis, pinealectomy, decreases both nocturnal and daily levels of melatonin (Brown *et al.* 1991) and associates with temporary hypertension (Zanoboni and Zanoboni-Muciaccia 1967), enhanced vasoconstriction (Cunnane *et al.* 1980) and increased incidence and prolonged duration of reperfusion arrhythmias (Sahna *et al.* 2002). The fact that hypertension induced by pinealectomy is reversible after exogenous melatonin administration suggests that it is at least partially mediated by the deficit of melatonin production after pinealectomy (Holmes and Sugden 1976). A different experimental model of decreased melatonin levels is the exposure of experimental animals to continuous 24 hours/day light (Delibas *et al.* 2002, Briaud *et al.* 2004, Paulis *et al.* 2005). This ´functional pinealectomy´ abolishes the nocturnal rise of melatonin concentration, without influencing day-time levels of melatonin (Brown *et al.* 1991). Although this method for induction of melatonin deficit represents a slightly more physiological approach, the effect of functional pinealectomy on ischemia-reperfusion injury, cardiac NOS activity and oxidative load has yet, to our knowledge, not been investigated.

In our experiments on hearts isolated from rats exposed to 4 weeks of continuous light, we observed increased incidence and severity and prolonged duration of

severe reperfusion arrhythmias in analogy to the proarrhythmic effect of surgical pinealectomy (Sahna *et al.* 2002). However, at the same time the course of recovery of all investigated functional parameters (coronary flow, left ventricular developed pressure, and contractility and relaxation index) during reperfusion was significantly improved. Having in mind the reported lower plasma levels of melatonin in light exposed animals, these results are in agreement with impaired post-ischemic recovery of contractility and with lower incidence of arrhythmias after the addition of melatonin in high doses to the perfusion solution in ischemia-reperfusion experiments (Važan *et al.* 2003).

Melatonin, whose level is decreased during the exposure of rats to continuous light, was reported to influence the major factors in the development of ischemia-reperfusion injury: the concentration of free radicals (Allegra *et al.* 2003, Reiter, 2000) as well as the intracellular calcium homeostasis (Mei *et al.* 2001). Especially the increased antioxidant capacity of the heart was suggested to contribute to mitigating effect of melatonin on size of infarct induced by I/R (Sahna *et al.* 2005).

In our study, the level of conjugated dienes, formed by membrane lipid peroxidation triggered by free radicals, after the exposure of rats to continuous light was only non-significantly elevated. On the other side continuous light attenuated the cardiac NOS activity. The inhibition of NOS activity could, at least partially, explain the controversy in the effect of continuous light on functional recovery and on reperfusion arrhythmias. The addition of NOS inhibitor before ischemia increased the incidence and the duration of reperfusion arrhythmias (Kawahara *et al.* 2003, Pabla and Curtis 1995). However, NOS inhibition improved the recovery of coronary flow (Kawahara *et al.* 2003) and contractility (Naseem *et al.* 1995) and decreased the size of infarct area (Woolfson *et al.* 1995). Supposedly, long-term decrease of NOS

activity in light exposed rats could lead to saving substrates for NO-production that allow more rapid restoration of NOS activity after ischemia, which represents a strong stimulus for NO production (Depre *et al.* 1997). The subsequent coronary artery dilatation may enhance the recovery of coronary flow, myocardial contractility and relaxation. On the other hand, more rapid changes in the myocardium caused by accelerated recovery of coronary flow could deteriorate electrical stability of the heart.

One should be aware, that lower melatonin levels levels may potentially result in attenuated antioxidant action of melatonin (Tan *et al.* 2000) and the lack of inhibitory effect of melatonin on glucocorticoid formation (Rebuffat *et al.* 1987), both facts being reported to inhibit NO formation (Maxwell, 2002) and decrease NOS expression (Maxwell, 2002, Wallerath *et al.* 1999).

Our study has shown that continuous light exposure may modulate the outcome of cardiac ischemia-reperfusion injury. However, further experiments are required to confirm whether observed changes are caused by decreased melatonin levels. These experiments should clarify whether observed effects are reversible by chronic melatonin treatment and to investigate correlations of above observed effects with glucocorticoid, melatonin and catecholamine plasmatic levels in rats exposed to continuous light.

We conclude that 4 weeks of continuous light exposure of rats improved ventricular functional recovery during reperfusion but increased the incidence, severity and duration of reperfusion arrhythmias in this group. In the left ventricle, the NOS activity was attenuated, but the concentration of CD was not significantly altered. Our results suggest that modulation of cardiac response to I/R by continuous light could be at least partially mediated by the attenuation of NO production.

Acknowledgements

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Tables

Table Pre-ischemic values of functional parameters

	Controls	Light
CF (ml/min)	9.23 ± 0.73	9.36 ± 0.63
LVDevP (mmHg)	87.07 ± 5.47	80.18 ± 4.36
+dP/dt max (mmHg/s)	3876 ± 277	3687 ± 196
-dP/dt max (mmHg/s)	1956 ± 193	1759 ± 188

Data are means ± SEM, CF, coronary flow, LVDevP, left ventricular developed pressure, +dP/dt max, contractility index, -dP/dt max, relaxation index.

Figure legends

Figure 1 Recovery of coronary flow (1A) and left ventricular developed pressure, LVdevP (1B) during reperfusion in controls and rats exposed to continuous light (light) for 4 weeks. Data are means ± SEM. *p<0.05, **p<0.01, ***p<0.001, Student t-test.

Figure 2 Recovery of contractility index, +dP/dt max (2A) and relaxation index, -dP/dt min (2B) during reperfusion in controls and rats exposed to continuous light (light) for 4 weeks. Data are means ± SEM. **p<0.01, ***p<0.001, Student t-test.

Figure 3 The incidence (3A) and summary duration (3B) of severe reperfusion arrhythmias: ventricular tachycardia (VT), ventricular fibrillation (VF) and sustained ventricular fibrillation (SVF) in controls and rats exposed to continuous light (light) for 4 weeks. Data are means ± SEM. *p<0.05, Student t-test.

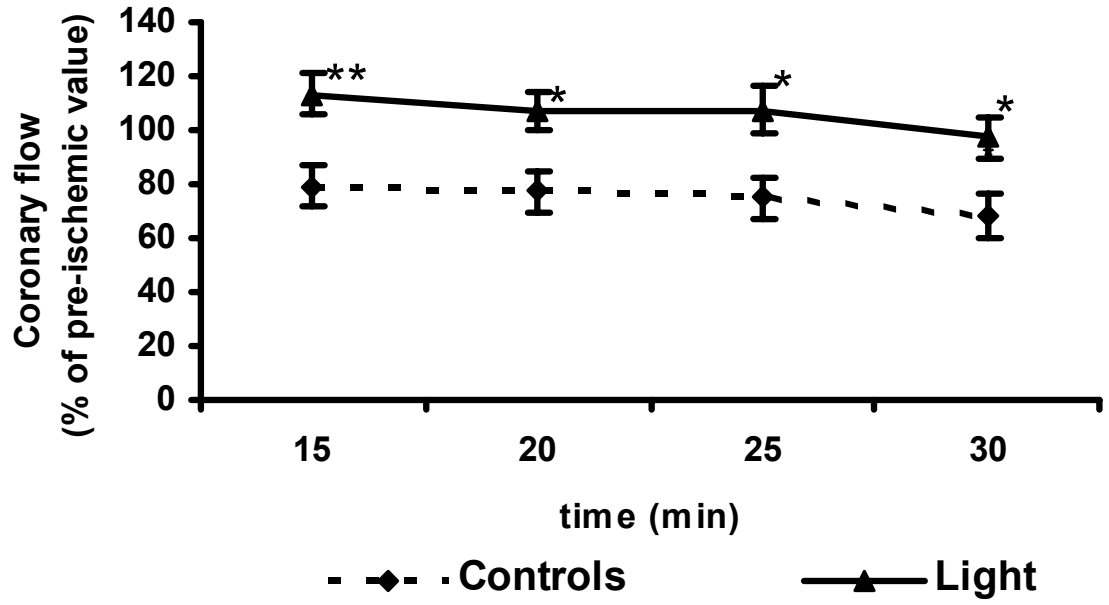
Figure 4 The arrhythmia score of reperfusion arrhythmias in controls and rats exposed to continuous light (light) for 4 weeks. Data are means ± SEM. *p<0.05, Mann-Whitney u-test.

Figure 5 The concentration of conjugated dienes, CD (5A) and the activity of NO-synthase, NOS (5B) in the left ventricle (LV) of controls and rats exposed to continuous light (light) for 4 weeks. Data are means ± SEM. *p<0.05, Student t-test.

Figures

Figure 1

1A



1B

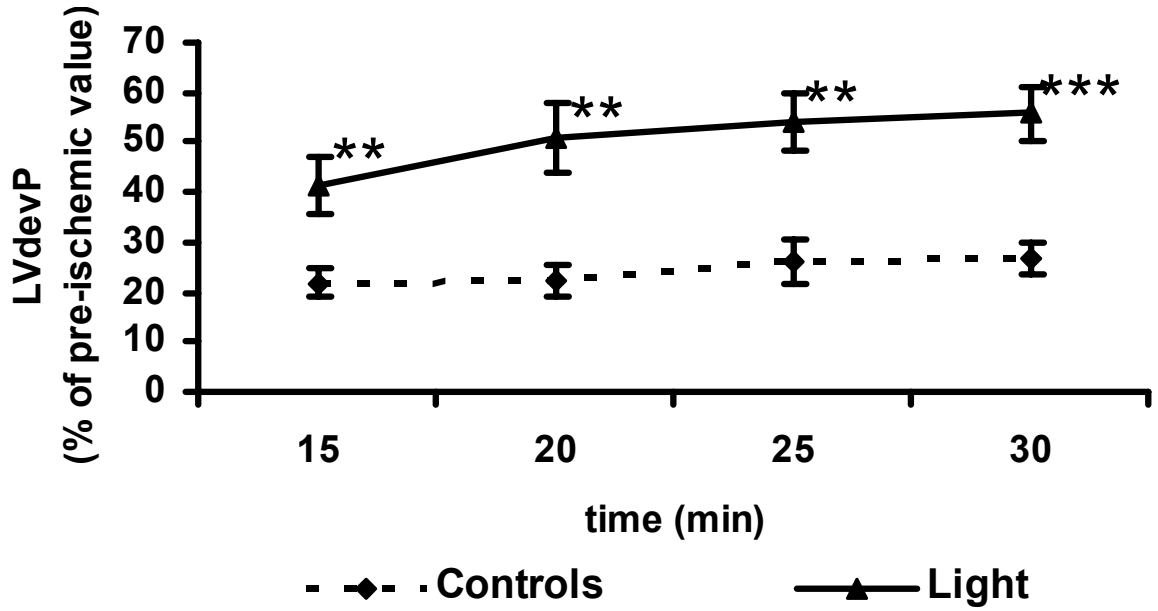
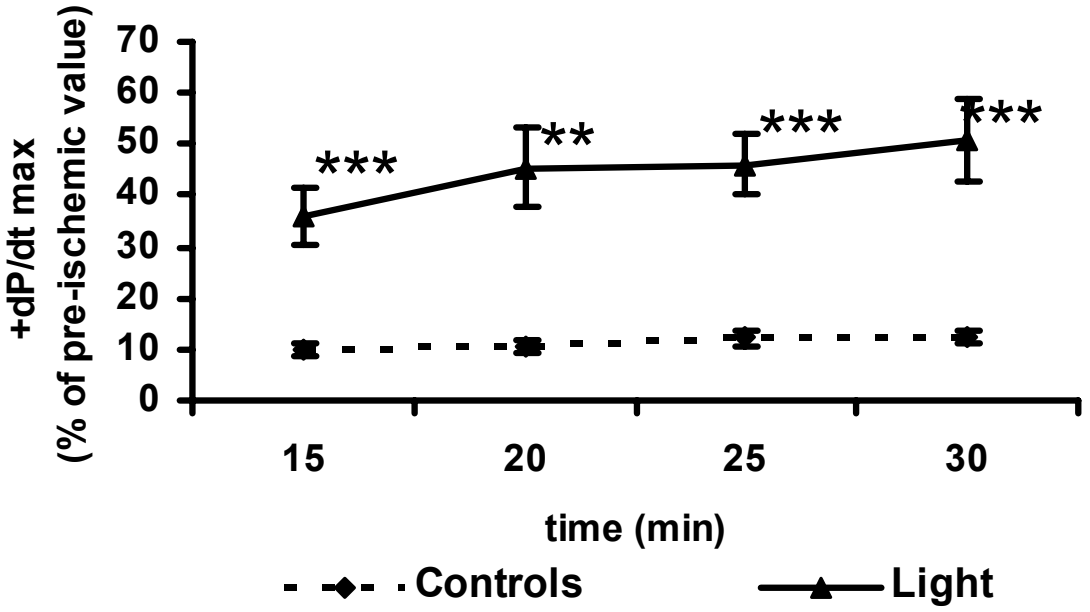


Figure 2

2A



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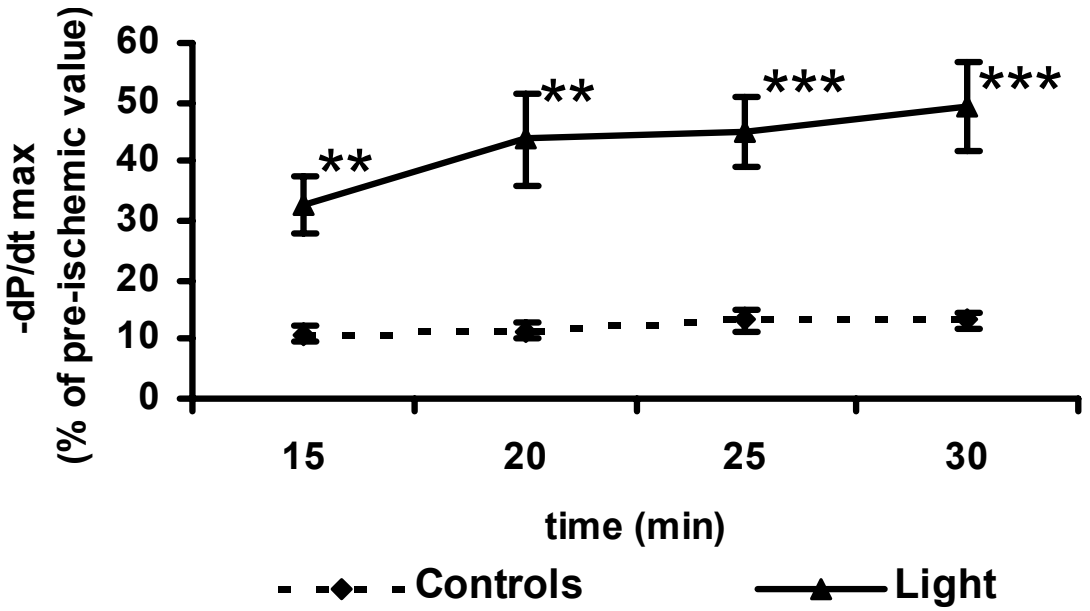
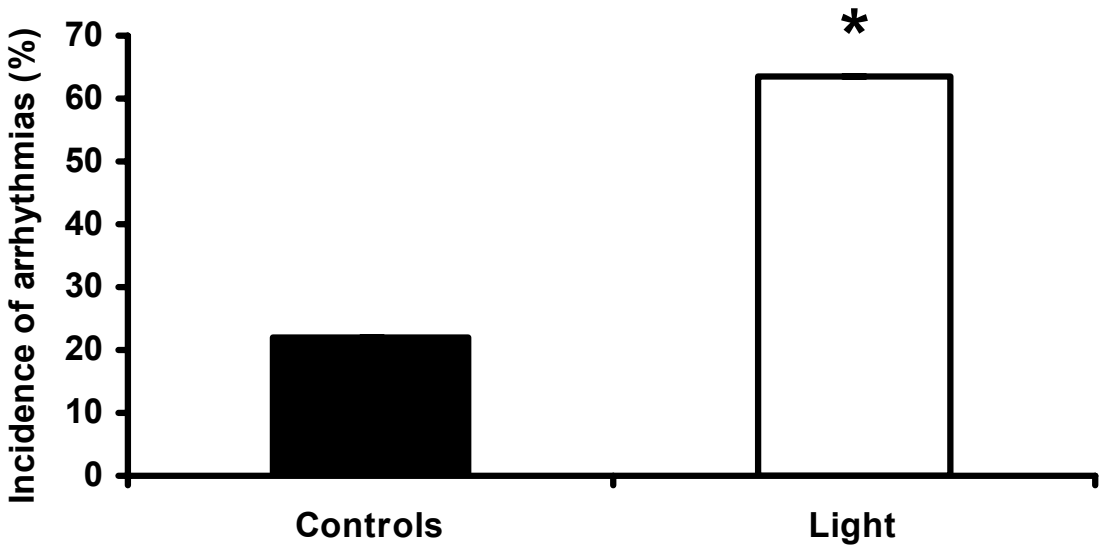


Figure 3

3A



3B

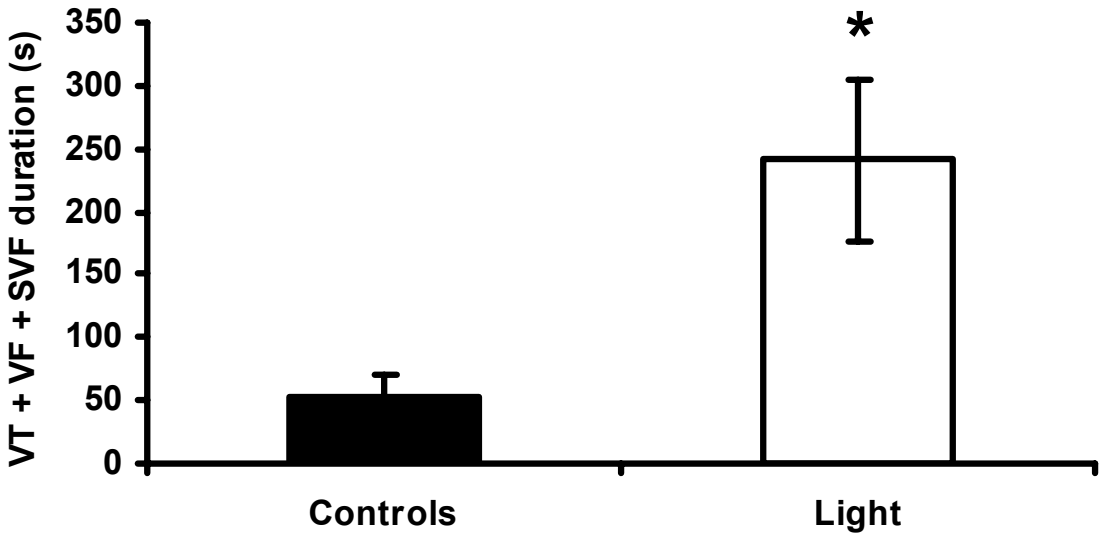


Figure 4

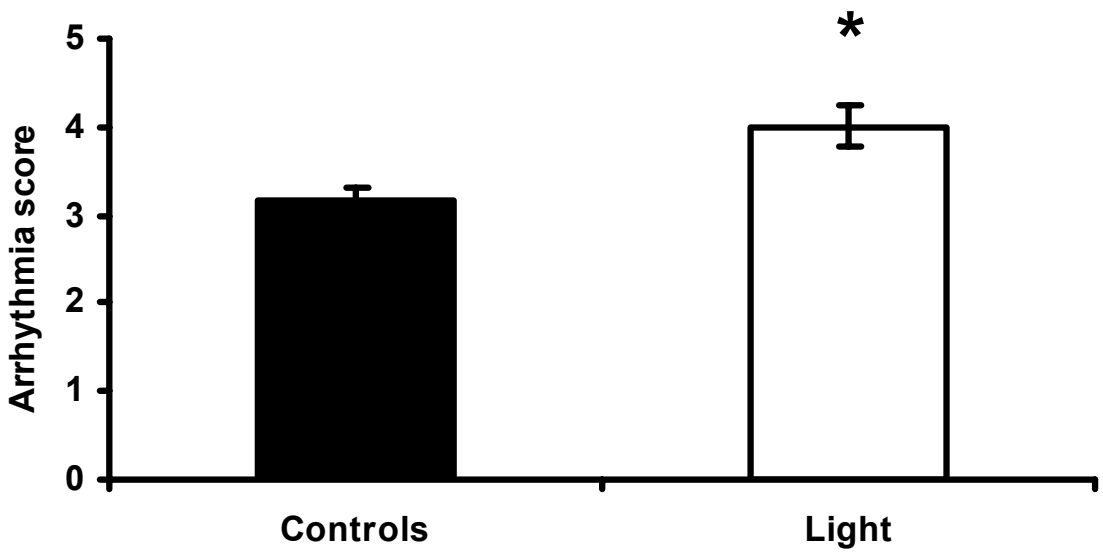
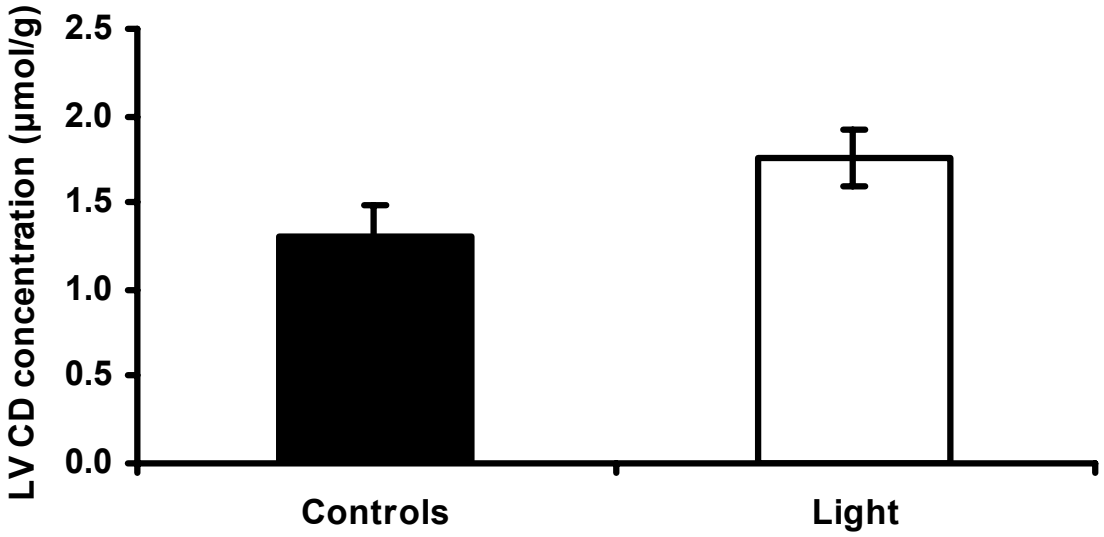


Figure 5

5A



5B