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Chronobiological Aspects of the Heart Rhythm Disorders at the Change of Pulmonary Ventilation in Rat Model

Pavol Svorc[1,2], Alexander Marossy[2], Pavol Svorc, Jr.[1],
Sona Gresova[2], Marek Buzga[1] and Benjamin L. Fulton[2]
[1]*Department of Physiology, Medical Faculty Ostrava University, Ostrava,*
[2]*Department of Physiology, Medical Faculty Safarik University, Kosice,*
[1]*Czech Republik*
[2]*Slovak Republic*

1. Introduction

Presently is well established, that most physiological functions of living organisms fluctuate with a circadian dependence. Many experimental and clinical studies have demonstrated that cardiovascular functions show a marked circadian rhythmicity (Smith et al., 1987; Henry et al., 1990). Circadian fluctuations occur both in blood pressure and heart rate, but also in the ocurrence of ventricular dysrhythmias, the onset of cardiovascular symptoms, and the manifestations of cardiovascular diseases.

Ventricular fibrillation is the most dangerous type of arrhythmia in humans and belongs to the group of the most frequent causes of sudden death after myocardial infarction. The development of ventricular fibrillation is strengthened by the difference between the duration of the refractory period and irregular electrical activity in the various parts of the heart. The probability of the development of such irregular activity is increased by an increased resting excitability, a decreased conduction velocity, and an increase in automaticity (Fisch, 1973; Opie et al., 1979; Carmeliet, 1988). The resistance of the heart to these disorders is dependent on its electrical stability, which can be measured by several parameters such as the duration of the vulnerable period (Wegria et al., 1941; Axelrod et al., 1975), ventricular flutter threshold (Szekeres & Papp, 1967), excitability threshold (Jones & Klein, 1982), or ventricular fibrillation threshold (Wegria et al., 1941; Gerst et al., 1966).

Factors that contribute to the development of various cardiac disorders not only include local myocardial ischemia (Ferrier et al., 1985; Saint et al., 1992), but also hypoxia (Nishimura et al., 1989) and respiratory and metabolic acidosis (Gerst et al., 1966; Rogers et al., 1973; Kujanik et al., 1984; 1985). It is generally accepted that some disorders of pulmonary ventilation belong to the group of proarrhythmogenic factors. The effect of systemic hypoxia, hypercapnia and acidosis (consequences of hypoventilation or an apneic episode) were investigated not only in experimental studies (Kujaník et al., 1984; 1985, Tomori et al., 1997; 2000) but also in clinical ones (Guilleminault et al., 1983; Peter, 1990; Kujanik et al., 2000a; 2000b).

Surprisingly, only a few studies have described the time of day that experiments were conducted or the synchronization of animals to external environmental periodicity such as the light-dark (LD) cycle. This can be a problem because the LD cycle represents one of the strongest circadian synchronizers of endogenous animal rhythms. For this reason, circadian variability should be considered an important factor especially in cardiovascular studies.

2. Circadian rhythms of the electrical stability of the heart at the changes of the pulmonary ventilation

2.1 Circadian rhythm of the electrical stability of the heart during normal ventilation

In the cardiovascular system, most physiological phenomena (such as heart rate, blood pressure, atrioventricular conduction, etc), pathological events (cardiac ischemia, infarction, sudden cardiac death, etc.) as well as non-invasive cardiac electrophysiological phenomena (heart rate variability, T-wave alternans, QT dispersion, etc.) have circadian rhythms (Guo & Stein, 2002). Data regarding circadian patterns in arrhythmias reported in the medical literature are unclear because the data derived from almost all of the studies were confounded by a variety of factors extraneous to intrinsic arrhythmogenic activity (Portaluppi & Hermida, 2007).

Knowledge regarding circadian variations in the electrophysiological properties of the heart is needed for more precise estimation of the risk of occurrence of ventricular arrhythmia. QT dispersion is considered to be an index of spatial inhomogenity of repolarization duration; increased dispersion of ventricular repolarization is believed to increase the risk of ventricular arrhythmia. Circadian variation of QT dispersion was detected in healthy subjects and in patients with uncomplicated coronary artery disease, with a peak value in the morning hours shortly after awakening (Bissinger et al., 2008; Hansen et al., 2008). In patients with heart failure or previous myocardial infarction (Hansen et al., 2008), or in patients with diabetes mellitus and coronary artery disease (Bissinger et al., 2008), circadian variation of QT dispersion was not detected. Gunez et al. (2008) found that P-wave dispersion (a new parameter for assessing the risk of atrial fibrillation) and QT dispersion do not show diurnal variation in patients with either ischemic or nonischemic heart failure treated with optimal drug therapy.

The dependence of the electrophysiological parameters of ECG on the changing of LD cycles was also confirmed in experimental studies. The circadian fluctuation of the electrical stability of the heart, measured by ventricular arrhythmia threshold (VAT), was followed during normal ventilation, hypoventilation and hyperventilation in pentobarbital-anesthetized rats after adaptation to a daily LD cycle of 12h:12h, with the dark period from 18:00h to 6:00h for 4 weeks. The VAT was estimated as the minimal amount of electrical current (in mA) needed for elicitation of ventricular arrhythmias and was measured directly by electrical stimulation of the heart (in open-chest experiments). The stimulating electrodes (diameter 1 mm and 5 mm inter-electrode distance) were fixed at the base of the right ventricle of rats positioned supine. Cardiac stimulation (rectangular pulses with a frequency of 30 Hz, impulse lenght of 10 ms, stimulation duration of 400 ms) was triggered by the initial pulse of the R wave. Current intensity was increased progressively by steps of 0,2 mA until ventricular arrhythmias were obtained. The parameters of stimulation were chosen to apply at least one of the impulses during the vulnerable period provided that the duration of the stimulation covered a minimum of 2 to 3 heart cycles. The ventricular arrhythmias

were of a mixed type with spontaneous mutual transitions between ventricular fibrillation, ventricular tachycardia and flutter which were comparable among the groups.
The 24h course of the VAT showed the highest susceptibility of the rat ventricular myocardium to arrhythmias between 12:00h and 15:00h and highest resistance between 24:00h and 03:00h under normoxic conditions (Svorc et al., 1994). Acrophase with confidence intervals were on -338^0 (-288^0; -7^0), in time at 22:53h (19:20; 00:28h) with mesor 2,59 ± 0,53 mA and amplitude 0,33 ± 0,11 mA (Figure 1).

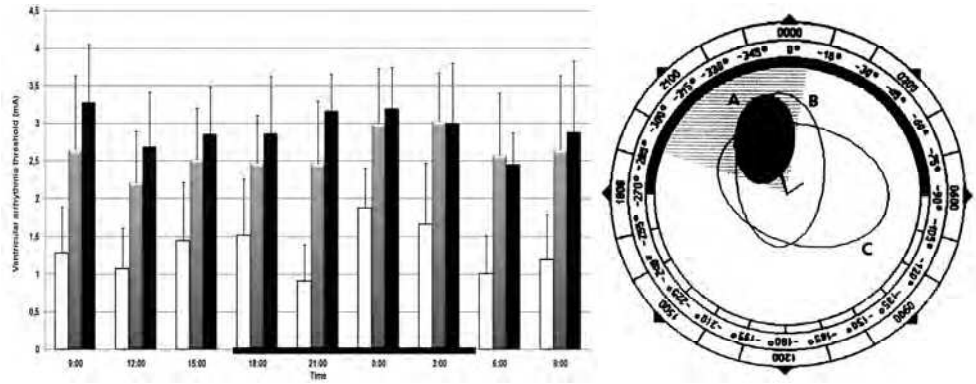


Fig. 1. Circadian rhythms of ventricular arrhythmia threshold during normal ventilation (gray columns), hypoventilation (empty columns) and hyperventilation (black columns); cosinor presentation of these rhythms during normoventilation (A), hypoventilation (B) and hyperventilation (C). Data are presented as mean ± SD. The dark bar indicates the dark cycle of the rat regime day.

Mechanisms responsible for the circadian changes in the vulnerability of the heart are probably multifactorial and are associated mainly with changes in electrophysiological properties of the myocardium. These are recognized as essential for the triggering and maintenance of arrhythmias. Portaluppi & Hermida (2007) summarized circadian rhythms of arrhythmia occurrence in humans, with peaks between 06:00h and 12:00h (ie. during the active part of the day). Similarly, during the day, when sympathetic output is enhanced and heart rate increased, P wave duration and its area, P-R interval, QRS duration and Q-T interval have been found to decrease. Estimated trough values usually occurred between 10:00h and 14:00h. During the night, following sympathetic withdrawal and parasympathetic dominance, the values of these electrical parameters increase, reaching their peak values between 12:00h and 06:00h. These changes are regulated mainly by the autonomic nervous system, which enables the heart to adapt to circadian fluctuations in demand by adjusting both its electrical activities and mechanical function (Guo & Stein, 2002).
In rats, the opposite tendency was observed. The highest vulnerability of the rat ventricular myocardium to arrhythmias occurred between 12:00h and 15:00h (non-active part of the day) and the highest resistance between 24:00h and 03:00h (active part). The possible mechanisms controlling the circadian rhythm of the VAT under normoxic conditions in rats can mainly be seen in the circadian alternations of the electrophysiological properties of the myocardium, which are determined to a large extent by a K^+ gradient (Fisch, 1973). Dispersion of duration of

the refractory period (QT interval) is the result of the action of more ion currents (Ca^{2+}, Na^{+}, Cl^{-} and inward rectifying K^{+} current) (Amitzur et al., 2000), which depends mainly on intracellular K^{+} concentration (Froldi et al., 1994). The incidence of ventricular arrhythmias directly correlates with serum K^{+} decreasing with a higher K^{+} concentration (Curtis et al., 1985; Winslow et al., 1989). In circadian dependence, the peak of minimal myocardial vulnerability to ventricular arrhythmias coincides with the peak of the maximal K^{+} serum concentration in rats (Stoynev et al., 1986; Poulis et al., 1989; Granda et al., 1996). The speed of impulse conduction from atria to ventricles (PQ interval duration) depends on action potential amplitude, reflecting the active role of Na^{+} channels (Carmeliet , 1988; Amitzur et al., 2000). Statistically significant sodium circadian rhythm occurs in the dark part of the rat regime day (Granda et al., 1996), which can increase vulnerability of the heart mainly to the arrhythmias originating from disorders of impulse production and conduction. Thus, the circadian pacemaker controlling the rhythm of serum K^{+} likely plays a key role in the circadian control of the VAT in rats under normal ventilatory conditions.

The next mechanism directly controlling the circadian rhythm of the electrical stability of the heart, involves the autonomic nervous system. Circadian rhythms in the autonomic nervous system activity are well known and constitute major triggers of cardiac arrhythmias. Increased sympathetic activity accelerates heart rate, favors spontaneous depolarization, shortens the effective ventricular refractory period, and decreases the threshold for ventricular fibrillation. In contrast, increased parasympathetic activity slows heart rate, decreases atrioventricular (AV) nodal conduction and in the presence of baseline sympathetic neural activity, increases both the ventricular refractory period and the ventricular fibrillation threshold (VFT) (reviewed in Portaluppi & Hermida, 2007). This direct and clear dependence, described in humans and in larger experimental animals, was not confirmed in rats (Svorc et al., 1994). The course and acrophase of the circadian rhythm of heart rate did not correspond either to the course or to the acrophase of the circadian rhythm of the VAT. Loss of heart rate dependence on the LD cycle refers to the fact that pentobarbital anesthesia probably minimizes or disturbs the effect of the LD cycle on heart rate under conditions of normal pulmonary ventilation. These results are consistent with results of Bruguerolle's group, who demonstrated the pertubations of daily rhythm of heart rate, locomotor activity and body temperature in rats but under ketamine anaesthesia. Total anaesthesia can probably modify the acrophase, mesor and amplitude of some rhythms but without the loss of the total rhythmicity (Prudian et al., 1997; Pelissier et al., 1998).

2.2 Circadian rhythm of the electrical stability of the heart during hypoventilation

Hypoxic states of the heart result from disproportionate amounts of oxygen supplied to cardiac cells and the amount actually required by the cell. The degree of hypoxic injury does not only depend on the intensity and duration of the hypoxic stimulus, but also on the level of cardiac tolerance to oxygen deprivation. Such oxygen deprivation can result from systemic hypoxia or local ischemia with consequences of two different mechanisms of action at the cellular level. Systemic hypoxia is usually a generalized phenomenon diffusely involving the whole myocardium, whereas ischemia is confined to the area supplied by the affected coronary artery. In ischemia, there is not only a drop in the supply of oxygen and other substrates, but also a significant reduction in the clearance of metabolites. In contrast, in ischemic hypoxia (often described as „cardiac hypoxia“) there is a combined action of both ischemia and hypoxia, while perfusion results in partial elimination of metabolites.

Ischemic hypoxia is clinically manifested primarily in ischemic heart disease (coronary artery disease) and its acute form, myocardial infarction, whereas systemic hypoxia is associated with chronic cor pulmonale of various origin, cyanosis due to a hypoxemic congenital heart disease, exposure to low barometric pressure (e.g. at high altitudes and ventilatory disorders) (Ostadal et al., 1999).

The effects of ventilatory disorders on the heart were broadly investigated in more experimental animal studies and under various experimental conditions. Failure of, or decrease in pulmonary ventilation is associated with systemic hypoxia, hypercapnia and acidosis resulting in various disorders of cardiovascular system activity.

There are some clinical trials describing the circadian rhythmicity of the cardiovascular events associated with changes in pulmonary ventilation. We cite the study by Kujanik et al. (2010) who referred to the incidence of the supraventricular and ventricular extrasystoles in healthy elderly men at low (200 m) and moderate altitude (1350 m) in the circadian dependence. The moderate altitude with the lower pO_2 shifted the highest occurrence of supraventricular and vetricular extrasystoles to the other times of day and increased the incidence of extrasystoles compared to low altitude by 2-fold. The authors concluded that the increase in extrasystole occurrence at high altitudes is probably caused by higher hypobaric hypoxia and resulting sympathetic drive. Healthy men at elevated altitudes show circadian and several ultradian rhythms of single ventricular extrasystoles dependent on the level of hypoxia.

Sleep, ventilatory disorders, especially obstructive or central sleep apnea (OSA or CSA), are associated with neurohormonal and electrophysiological abnormalities that may increase the risk of sudden death from cardiac causes, especially during sleep. Gami et al. (2005) followed this dependence in 112 subjects who died suddenly from cardiac causes. They found that from midnight to 06:00h, sudden death from cardiac causes occurred in 46% of patients with OSA compared with 21% of individuals without OSA. Patients who experienced sudden death from cardiac causes from midnight to 06:00h, had a significantly higher apnea-hypopnea index than those with sudden death from cardiac causes during other intervals, and the apnea-hyponea index correlated directly with the relative risk of sudden death from cardiac causes from midnight to 06:00h. Thus, individuals with OSA experience a peak in sudden death from cardiac causes during the hours of sleep, which contrasts strikingly with the nadir of sudden death from cardiac causes during this period in people without OSA. Variation in the onset of myocardial infarction was found in patients with and without OSA. Myocardial infarction occurred between midnight and 06:00h in 32% of OSA patients and 7% of non-OSA patients. Of all patients who experienced a myocardial infarction between midnight and 06:00h, 91% had OSA (Kuniyoshi et al., 2008). These findings suggest that OSA may be a trigger for myocardial infarction in patients who experience nocturnal onset of myocardial infarction should be evaluated for OSA. Future research should address the effects of OSA therapy for prevention of nocturnal cardiac events. These studies refer to the fact that circadian rhythmicity may have practical relevance in screening for patients with OSA and may have prognostic clinical value in predicting future cardiovascular events (Gami et al., 2005; Kuniyoshi et al., 2008).

In experimental animal models, the link between disorders of pulmonary ventilation and the incidence of ventricular arrhythmias was also demonstrated in the circadian dependence. Otsuka & Watanabe (1990) followed the circadian rhythms of three types of bradyarrhythmia incidence in rats. The 24h chronogram of bradyarrhythmia incidence

showed 2 peaks: the higher peak between 05:00h and 09:00h (immediately after the start of the light cycle) and the second one between 11:00h and 18:00h. The hourly distribution of the apnea index coincided with the highest peak of the 24h chronogram of bradyarrhythmia incidence in rats. In ketamine/xylazine-anesthetized rats from experiments performed by Bacova et al. (2010), RR and PQ interval duration showed the significant LD differences, except in the QT and QTc interval in spontaneously breathing animals. The initial significant LD differences in PQ interval and loss of dependence on LD cycle in the QT interval were preserved during short-term asphyxia induced by apneic episode (30 s to 60 s). In contrast, long-term asphyxia (90 s to 120 s) eliminated LD dependence in the PQ interval; however, significant LD differences were shown in the QT interval. It was concluded that myocardial vulnerability was dependent not only on changes in pulmonary ventilation but also on the LD cycle.

In our hypoventilatory rat model, hypoventilation-induced systemic hypoxia, hypercapnia and acidosis decreased the VAT and heart rate values in all measured intervals during a 24h period. The mesor (1, 33 mA), amplitude (0,14 mA) was decreased, and the circadian rhythm of the VAT was changed to biphasic with a smaller peak between 15:00h and 18:00h and higher peak between 24:00h and 03:00h. The hypoventilatory circadian rhythm of the VAT was not significant as revealed by the population mean cosinor (Svorc et al., 1997, 2000a) (Figure 1).

The decreased electrical stability of the heart during the course of the entire 24h period confirmed results from other electrophysiological studies investigating the effect of hypoxia on myocardium. The duration of the action potential was significantly decreased at the start of hypoxia in isolated hearts of rats (Perchenet and Kreher, 1995), rabbits (Baker et al., 2001), cats (Vleugels et al., 1980), guinea pigs (Sanguinetti et al., 1988) and dogs (Ferrier et al. 1985). The phase plateau of action potential shortened, and ATP content decreased (Noma, 1983). In isolated rabbit AV preparations, hypoxia impaired AV nodal conduction and depressed automaticity (Nishimura et al., 1989). In *in vivo* rabbit models, the sinus interval was gradually increased with duration of hypoxia. Atrio-His interval and His-ventricular intervals were prolonged (Sawanobori et al., 1995). The membrane potential was decreased, excitability and impulse conduction between Purkinje fibres and muscle tissue were depressed in isolated Purkinje fibres of papillary muscle from dogs (Ferrier et al. 1985).

In experiments using cats, hypercapnic hypoxemia produced N_2 inhalation and evoked all types of conduction blockades, supraventricular extrasystoles, peaked T waves, elevated ST segments and decreases in R waves (Tomori et al., 1997; 2000). In experiments conducted by Gerst et al. (1966) using dogs, pH changes owing to respiratory acidosis and alkalosis did not affect the electrical stability of the heart, measured by VFT. Neither respiratory acidosis nor hypoxia alone significantly changed the VFT, but together they increased the followed parameter in the canine ventricle (Rogers et al., 1973). Kujanik et al. (1985) described dynamic changes of the VFT in rats with various types of ventilation, but not its circadian dependence. The VFT was decreased during mild hypoxia and acidosis and increased during serious hypoxia and acidosis. The vulnerable duration period was prolonged during hypoventilation.

These disorders can be explained by a sudden increase in extracellular K^+ concentration, which plays a crucial role in the changes in resting membrane potential, and can produce ectopic activity as well as inhibition of the rapid reaction (Opie et al., 1979). The rapid increase in extracellular K^+ concentration is the result of K_{ATP} channel activation. It is

inactivated in normoxic conditions, but it is activated in hypoxic or anoxic conditions (Noma & Shibasaki, 1985; Sanguinetti et al., 1988; Daut et al., 1990; Billman et al., 1993). During ischemia, activation of K_{ATP} channels limits Ca^{2+} input into metabolically stressed cells. In ischemic regions, it can lead to the dispersion of refractory periods between normal and ischemic myocardium; thus, the blockade of K_{ATP} channels acts as an antiarrhythmic (Wolleben et al., 1989). On the other hand, no correlation between total ATP concentration and the electrical activity of the heart was found during relative hypoxia or ischemia of the myocardium (Kreher & Wedetti, 1986). We can suppose that the mechanism of K^+ current activation by hypoxia can also be responsible for changes in the electrical stability of the heart in circadian dependence, although the biphasic course of VAT is not specifically explained by this mechanism.

The control mechanisms responsible for the biphasic circadian course in the electrical stability of the heart during hypoventilation are not known, they are probably multifactorial and are mainly associatedwith changes in the electrophysiological properties of myocardium in hypoxic conditions. The biphasic course can be partly explained by the effect of histamine on rat ventricular arrhythmias under hypoxic conditions. Dai (1989) demonstrated that hypoxia and histamine can increase susceptibility to arrhythmias. If hypoxia does not alter the circadian rhythm of blood histamine levels, then increased myocardial susceptibility to arrhythmias can be in the certain range influenced by histamine under the hypoxic conditions. Our results from a hypoventilatory rat model support this hypothesis. The very close relationship is between rhythm of the electrical stability of the heart and the rhythm of histamine concentration in the blood. In a rat model, Catini & Legnaioli (1992) showed (upon synchronization to a natural lighted regime) that circadian oscillations in histamine concentration in the blood and in the thyroid gland are biphasic, with peaks at 07:50h and at 19:50h, in time the lowest VAT.

2.3 Circadian rhythm of the electrical stability of the heart during hyperventilation

In our experiments, hyperventilation increased the VAT at each measurement interval, but did not change the characteristic of its circadian rhythm. The 24h hyperventilatory rhythm of the VAT was non-significant, acrophase was shifted to -40^0 (02:40h), mesor was increased (2,91 mA) and amplitude was decreased (0,13 mA) (Figure 1) (Svorc et al., 2002). Although the results are not unequivocal, these ventilatory changes probably have a causal relationship with disorders of ion kinetics and/or ion distributions inside and outside of myocardial cells, and also with circadian dependence. From what is currently known, if the electrical stability of the heart is dependent on ion concentration changes, it follows that circadian rhythm of the VAT most probably behaves similarly to the circadian rhythms of the single ions. The unanswered question is how does light and dark act on ion kinetics ventilatory disorders and on return to normal ventilatory conditions, after synchronization to a 12h:12h LD regime?

3. The electrical stability of the heart in a hypoventilation/reoxygenation model

The onset and development of ventricular arrhythmias depends on many factors to which some disorders of pulmonary ventilation also belong. However, not all consider the effect of the recovery of oxygen delivery (reoxygenation) after hypoxic episodes to be the onset or development of ventricular arrhythmias. Reoxygenation after hypoxic episodes does not

automatically normalize myocardial properties (electrophysiological and mechanical), but can increase the risk of onset of reoxygenation arrhythmias (Winslow et al., 1983; Perchenet & Kreher, 1995; Bilinska et al., 1996; Bernauer, 1997; Mubagwa et al., 1997; Shinmura et al., 1997; Guo et al. 2005).

Tissue hypoxia, for example in patients with sleep apnea, is an important factor in heart disease (Yokoe et al., 2003). Hypoxia and reoxygenation expose the myocardium to extremes in redox stress, which can result in the initiation of a series of cellular pathways leading to tissue injury and death. Myocardial hypoxia reduces left ventricular contractile performance (Tanonaka et al., 1989; Draper & Shah, 1997; Jeroudi et al., 1994; Kang et al., 2000); however, recovery of the contractile force was less than 10% and recovery of the myocardial high-energy phosphates during reoxygenation was approximately 40% (Tanonaka et al., 1989).

The study by Pahor et al. (1989) demostrated the antiarrhythmic effect of verapamil on spontaneous ventricular arrhythmias during reoxygenation after 15 min of glucose-free hypoxia and on programmed electrical stimulation-induced ventricular fibrillation in isolated Langendorff-perfused guinea pig hearts. Verapamil added during reoxygenation reduced the incidence of reoxygenation arrhythmias and ventricular fibrillation, but it had no effect on programmed stimulation-induced ventricular fibrillation. It is likely that verapamil exerts its antiarrhythmic effect by preventing cellular calcium overload during hypoxia and reoxygenation.

Hypoxia and mild acidosis progressively diminished the amplitude and duration of these slow-response action potentials, whereas reperfusion/reoxygenation progressively increased their amplitude even more than that in the control (prehypoxic value). Action potential duration increased (at all levels) during reperfusion compared with that in hypoxia and mild acidosis; however, action potential duration remained shorter than the control (prehypoxic level). The effects of hypoxia (and mild acidosis) and subsequent reoxygenation seem similar to the effects of elevating extracellular kalcium levels (increased inward current). From these experiments, one cannot, however, distinguish the effects of hypoxia on the inward currents from those on the outward currents (Bhattacharyya & Acharya, 1988). The response of hypoxic and acidotic ventricular muscle tissue to subsequent reoxygenation was studied by Bhattacharyya et al. (1991). Ventricular muscle tissue exhibited the different response to reoxygenation after hypoxia and acidosis: (1) arrhythmias, without much depolarization of the membrane potential; (2) oscillatory afterpotentials during the late diastole, which lessened in amplitude as the time of reoxygenation increased, but no arrhythmias; or (3) a pronounced slowed phase of repolarization (hump), but no arrhythmias. These different effects of reoxygenation did not occur if the concentration of K^+ in hypoxic and acidotic ventricular muscle tissue was much higher than 4.6 mM. Common to these three different responses was the prolongation of the action potential duration during reoxygenation at the 50% and 90% levels of repolarization (APD_{50} and APD_{90}) and a slight increase in the resting tension after 30 to 40 min. of reoxygenation.

Membrane potential changes of atrial fibroblasts in response to mechanical stress have been considered to modulate the rhythmic electrical activity of healthy hearts. It is suggested that cardiac arrhythmia after infarction is related to enhanced susceptibility of fibroblasts to physical stretch. It indicates that transmembrane currents in atrial fibroblasts are sensitive to changes in tissue oxygenation and altered electro-mechanical function of the ischemic heart may involve changes in the membrane potential of cardiac fibroblasts (Kamkin et al., 2003).

The proarrhythmogenic effect of reoxygenation was confirmed by several studies using various agents. For example, in the papillary muscles of guinea pigs, Ca^{2+} entry through Ca^{2+} channels apparently synchronized Ca^{2+} release from the sarcoplasmic reticulum, and a high concentration of D-600 apparently decreased the incidence of arrhythmias. Tetrodoxin and nicorandil decreased arrhythmias, probably by decreasing the Na^+ current or by increasing the ATP-sensitive K^+ current, respectively (Hayashida et al., 1996). Thus nicorandil antagonizes the cellular mechanisms that underlie the reoxygenation arrhythmias and prevent reoxygenation-induced arrhythmias (Xu et al., 1993). The effects of a selective blocker of Ca^{2+} influx by Na^+/Ca^{2+} exchange, KB-R7943, on the reoxygenation-induced arrhythmias and the recovery of developed tension after reoxygenation, were investigated in guinea pig papillary muscles. This blocker selectively inhibited the reverse mode of Na^+/Ca^{2+} exchange, attenuated reoxygenation-induced arrhythmic activity and prevented contractile dysfunction in guinea pig papillary muscles. These results suggest that Ca^{2+} influx by Na^+/Ca^{2+} exchange may play a key role in reoxygenation injury (Mukai et al., 2000).

It is well established that hypoxia followed by reperfusion may be fatal and result in generation of reactive oxygen species (ROS) and subsequent tissue damage (Danielsson et al., 2007) and is associated with additional damage to the myocardium by oxidation of cellular components and activation of the inflammatory cascade (Cerniway et al., 2002). Some of isoflurane's cellular actions, such as interference with intracellular Ca^{2+} handling, inhibition of the respiratory chain, and the capability to produce oxygen radicals, could result in impaired cellular function during ischemia/reoxygenation. When isoflurane was applied during ischemia/reperfusion, intracellular Ca^{2+}, oxygen radical formation, arrhythmic events, and contractile function were increased in rat cardiomyocytes. Furthermore, increased oxygen radical generation was detected in isoflurane-treated myocytes during reoxygenation. Isoflurane given during ischemia/reperfusion in a study by Dworschak et al. (2004) induced intracellular Ca^{2+} accumulation and impaired cell function. These potentially harmful effects were associated with diminished Ca^{2+} clearance and accelerated oxygen radical production. In clinical practice, reperfusion of ischemic myocardium usually occurs under high arterial oxygen levels. However, this might aggravate cardiac ischemia/reperfusion injury caused by excessive oxidative stress. In an experimental *in vivo* study, the cardioprotective role of hypoxic reoxygenation during initial reperfusion was assessed. Hypoxic reoxygenation at the onset of reperfusion attenuated myocardial ischemia/reperfusion injury and helped to preserve cardiac performance after myocardial ischemia in a pig model (Abdel-Rahman et al., 2009).

The mitochondrial K_{ATP} channel (mitoK_{ATP}) opening which can be triggered by activation of the angiotensin II (Ang II) type 1 receptor on ischemia/reperfusion causes ROS-induced ROS release. The electrophysiological actions of Ang II linked with the genesis of reperfusion arrhythmias were elucidated by clarifying the roles of Ang II and mitoK_{ATP} on cardiac impulse propagation. MitoK_{ATP} blocker and AT1 receptor blocker abolished conduction block and conduction delay induced by Ang II. This result demonstrated that a mitoK_{ATP} channnel blocker protectively associated with arrhythmogenesis properties during reoxygenation **(**Wakatsuki et al., 2009**).** The inhibition of inducible nitric oxide (NO) synthese (NOS) raises the peroxidative and apoptotic level in the hypoxic heart indicating that this isoform may have a protective effect on this organ against hypoxia/reoxygenation injuries, and challenges the conventional wisdom that isoforms of NOS are deleterious under these conditions. These findings could help in the design of

new treatments based on NO pharmacology against hypoxia/reoxygenation dysfunctions (Rus et al., 2011).

In contrast to previous evidence about the harmful effect of reoxygenation on myocardium, Milano et al. (2010) refer to the protective effect of chronic hypoxia against ischemia/reperfusion damage. In rat experiments, exposure to chronic hypoxia results in impairment of myocardial tolerance to ischemia/reperfusion, greater injury and reduced recovery of performance. Daily reoxygenation markedly reduced hypoxia-induced derangements by accelerating intrinsic adaptive changes in the myocardium. These findings correlate with enhanced NO signalling via up-regulation of the endothelial isoform of NOS.

Studies investigating the effect of ischemia/reperfusion or hypoxia/reoxygenation on the onset and development of ventricular arrhythmias concentrate mainly on the temporally current mechanical and metabolic changes in myocardial cells, often without respect to circadian dependence. The question remains whether vulnerability of the ventricles to arrhythmias is primarily changed only by the factors resulting from altered ventilation, or are there also natural factors (eg, environmental periodicities) that can influence the parameter being studied?

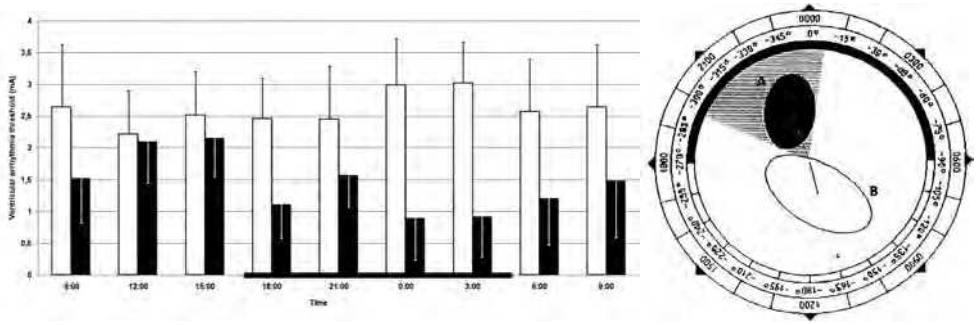


Fig. 2. Circadian rhythms of the ventricular arrhythmia threshold during normoventilation (empty columns) and reoxygenation after hypoventilation (black columns); cosinor presentation of these rhythms during normoventilation (A) and reoxygenation (B). Data presented as mean ± SD. The dark bar indicates the dark cycle of the rat regime day.

The analysis of VAT circadian rhythms was performed in a hypoventilation/reoxygenation group, in which pentobarbital anaesthetized animals were subjected to 20 min. of hypoventilation followed by 20 min. of reoxygenation. Reoxygenation expressively altered the VAT circadian rhythms inversely compared to the control group. Biphasic character was kept only after 5 min. of reoxygenation. 10, 15 and 20 min. of reoxygenation gradually changed the VAT circadian courses to inverse ones with the highest values between 12:00h and 15:00h and lowest values between 24:00h and 03:00h. The mesor was decreased (1,41 mA), amplitude was increased (0,57 mA) and acrophases was on -165^0 (Figure 2).

A more detailed analysis of the circadian VAT changes after 5., 10., 15. and 20. min. hypoventilation showed that the acrophases from 10., 15. and 20 min, of hypoventilation were nonsignificantly shifted compared to 5 min. of hypoventilation (Table 1). The characteristic biphasic course of the circadian rhythms of VAT was seen only after 10 min. of hypoventilation (Figure 3) (Svorc et al., 2000b, 2002).

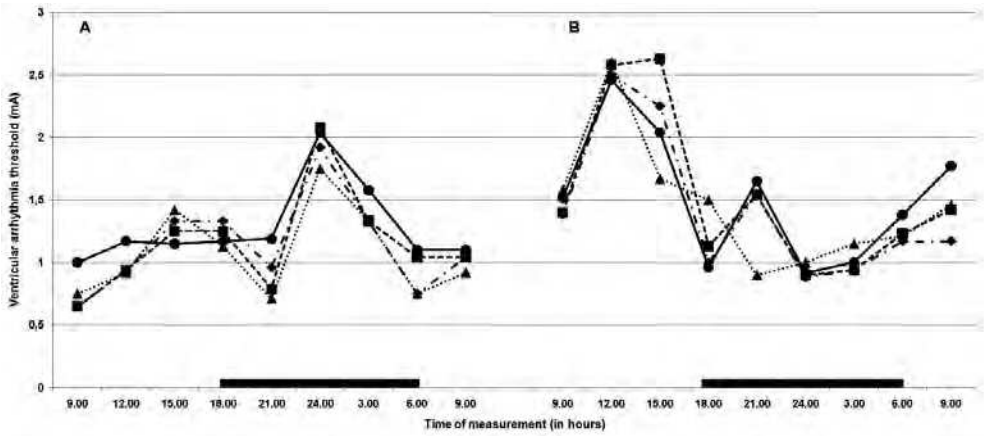


Fig. 3. Circadian rhythms of the ventricular arrhythmia threshold from hypoventilation/reoxygenation model after 5 min. (circles), 10 min. (squares), 15 min. (rhombus) and 20 min. (triangles) of the respective ventilation. A – initial hypoventilation, B – subsequent reoxygenation. The dark bar indicates the dark cycle.

	Initial hypoventilation				**Subsequent reoxygenation**			
	5 min.	10 min.	15 min.	20 min.	5 min.	10 min.	15 min.	20 min.
Mesor	1.32±0.1	1.17±0.2	0.15±0.2	0.12±0.1	1.41±0.1	1.41±0.1	1.29±0.1	1.42±0.1
Amplitude	0.33±0.2	0.33±0.2	0.35±0.2	0.20±0.2	0.41±0.2	0.48±0.1	0.37±0.2	0.53±0.1
Acrophase								
in degrees	-356±25	-11±37	-36±30	-30±58	-166±24	-165±17	-172±29	-156±10
in hours	23:50±1.40	00:44±2.28	02:24±2.00	02:50±2.32	11:04±1.36	11:00±1.08	11:28±1.56	10:24±0.4

Table 1. Parameters of the circadian rhythms of the ventricular arrythmia threshold (VAT) in a rat model of hypoventilation/reoxygenation.

Results from an experimental study involving ketamine/xylazine-anaesthetized rats (Svorc et al., 2005) indicated that although the electrical stability of the rat heart did not demostrate a dependence on LD cycle during normal pulmonary ventilation (probably an effect of ketamine/xylazine anaesthesia), hypoventilation/reoxygenation changed myocardial vulnerability by a manner dependent on LD cycle. It appears that rat myocardium is probably more sensitive to systemic asphyxia induced by hypoventilation and reoxygenation during the light (non-active) part of the day (Figure 4).

Hypoventilation and recovery of pulmonary ventilation produce different myocardial responses to electrical stimulation of the heart in individual animals. The reactions of animals to electrical stimulation under different ventilation conditions in both light parts of the day are shown in Table 2. A X^2-test was performed on the basis of these individual responses in the aspect of previous threshold values for evaluating the effect on the LD cycle. The significant effect of the LD cycle on the VAT changes was conformed for the period of hypoventilation ($p < 0,05$) as well as reoxygenation ($p < 0,01$), respectively.

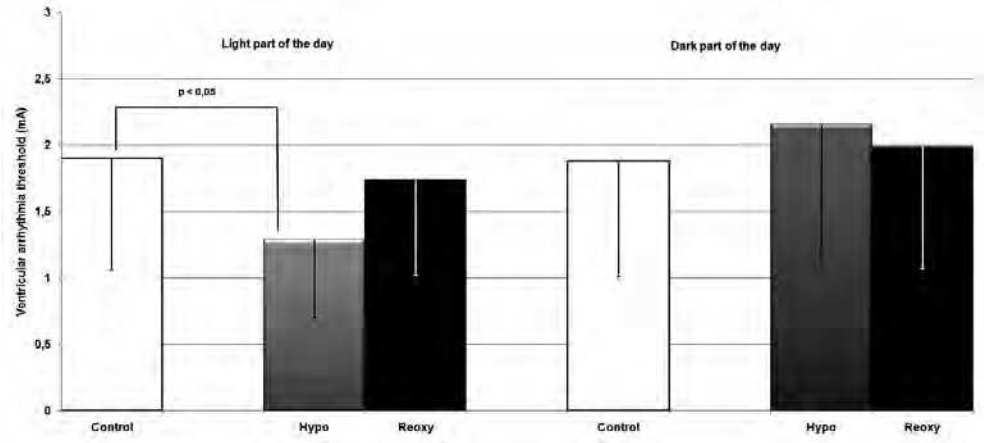


Fig. 4. The changes of the VAT in the hypoventilation (Hypo)/reoxygenation (Reoxy) rat model in the light and dark part of the regime day. Control – VAT value after the surgical interventions (tracheotomy, thoracotomy) and 5 min. stabilization at the parameters of normal artificial pulmonary ventilation (empty column – light and dark group). Hypo – the average VAT value from measurement after 5, 10, 15 and 20 min. of hypoventilation (gray columns). Reoxy – the average VAT value from measurement after 5, 10, 15 and 20 min. of reoxygenation (black columns).

	Hypoventilation vs. control		**Reoxygenation vs. hypoventilation**	
	Light	**Dark**	**Light**	**Dark**
VAT decrease	10/11 (90,9 %)	7/19 (36,8 %)	2/12 (18,2 %)	13/19 (68,4 %)
VAT increase	1/11 (9,1 %)	12/19 (63,2 %)	9/11 (81,8 %)	6/19 (31,6 %)

Table 2. Individual responses of animals to electrical stimulation of the heart. Numerator - number of animals with VAT changes against the previous VAT measurement, denominator - number of animals in experimental group.

As mentioned above, alterations in myocardial vulnerability depend mainly on changes in ion concentrations. LD differences in the electrical stability of the heart might reflect the LD differences in ion concentrations. In hypoxia/reoxygenation or ischemia/reperfusion models, more authors describe myocardial Ca^{2+} accumulation. Ca^{2+} overload in myocytes is one of the many causes of the reperfusion injury (Kamiyama et al., 1996; Mubagwa et al., 1997; Shinmura et al., 1997; Sharikabad et al., 2000). It was hypothetized that the delayed afterdepolarizations producing the substrate for arrhythmogenesis of the serious ventricular dysrhythmias (Ca-mediated, non re-entry arrhythmias) are the result of such Ca^{2+} overload (Whalley et al., 1995). Sharikabad et al. (2000) describe unchanged intracellular concentration of Ca^{2+} ions during hypoxia, but concentrations were 3 to 4 times higher during reoxygenation in isolated rat hearts. During reoxygenation of hypoxic rat cardiomyocytes there is a correlation between extracellular Ca^{2+} and ROS (the second factor involved in ischemia/reperfusion-induced cardiomyocyte damage), whereas the correlation between cell Ca^{2+} and ROS levels is less consistent. These results indicate that ROS levels during oxidative stress are at least partly dependent on extracellular Ca^{2+} concentration, but

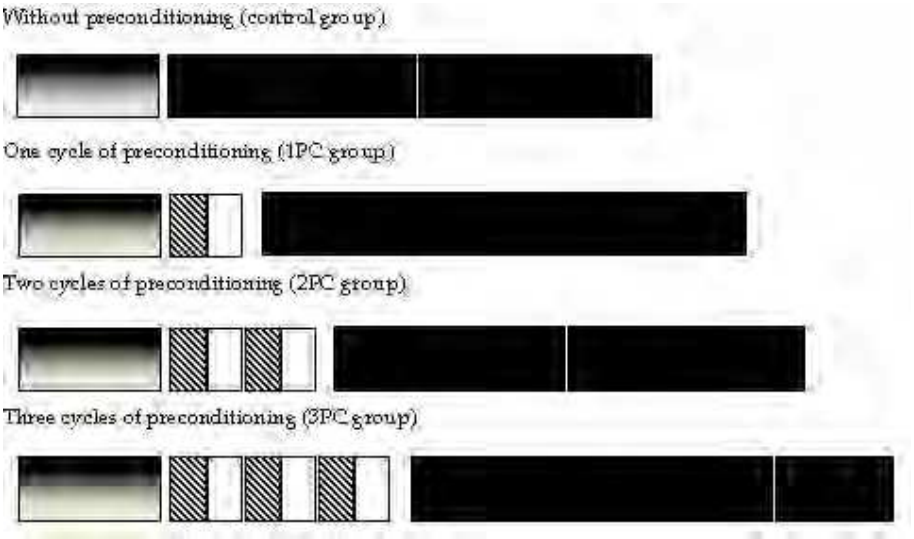
ROS (H_2O_2) can increase or decrease cardiomyocyte Ca^{2+} accumulation during reoxygenation in a concentration-dependent manner (Sharikabad et al., 2004). Decrease of intracellular pH can also participate in the mechanism of myocardial reoxygenation damage. This decrease is mediated, at least in part, by anion exchange stimulation (Cl^-/HCO_3^- exchange) through protein kinase C activation. This exchange takes part in the reoxygenation-induced Ca^{2+} overload and in contractile dysfunction (Kawasaki et al., 2001).
The decrease in electrical stability of the heart can also be the result of cellular K^+ loss during hypoxia (Shivkumar et al., 1997). Hypoxia (Perchenet & Kreher, 1995) significantly decreases action potential duration probably through the activation of the K_{ATP} channels and increased K^+ ion efflux. The inhibition of the outward K^+ currents showed the cardioprotective effect during reperfusion (Liu et al., 1993; Tosaki et al., 1996). The increase of intracellular concentration of Na^+ ions in myocardial cells, and Ca^{2+} overload, can contribute to the rise of reoxygenation arrhythmias (Takeo et al., 1995; Kamiyama et al., 1996; Shinmura et al., 1997).
Systemic hypoxia induced by hypoventilation changed the electrical stability of the rat heart in dependence on the LD cycle. Although the VAT decreased parallelly in both light parts of day during 20 min. hypoventilation, it was demonstrated that 1. The significant higher average VAT values were in the dark part of the day (active phase) versus the light part (non-active phase); 2. Rat hearts are more resistant to systemic hypoxia in the dark part of the day; and 3. The significant decrease of the VAT refer to the proarrhythmogenic effect of the systemic hypoxia only in the light part of day. These differences are probablya result of the changed myocardial reactivity to electrical stimulation dependent on the LD cycle.
Although reoxygenation returned VAT level to that of control values in both light (non-active) and dark (active) parts of the day, the problem remains that the VAT was significantly increased versus hypoventilatory value only in the light part of the day. The contrary tendency was found in the dark part of the day. The decrease in the dark part of the day probably signals the larger extent of the reoxygenation injury or increased sensitivity of the myocardium to the ventricular arrhythmias in the dark part of the day. This fact is supported by our previous results in rats under pentobarbital anaesthesia, where the nadir of the VAT circadian rhythm was found between 24:00h and 03:00h during reoxygenation (Svorc et al., 2000a).
The significant hypoventilatory LD differences in the thresholds show the different LD effects of hypoventilation–induced systemic asphyxia on the electrical stability of the rat heart. The higher values in the dark part of the day are probably the result of varying myocardial sensitivity to systemic asphyxia in the LD dependence, although there are more reports referring to the depressive effect of hypoxia on the circadian rhythms in rats (Bishop et al., 2000; 2001; Fenelon et al., 2000; Mortola & Seifert, 2000), in golden hamsters (Jarsky & Stephenson, 2000), and in humans (Bosco et al., 2003). An important and still unanswered question remains: whether the mechanisms responsible for altered myocardial vulnerability are mobilized mainly by hypoventilation-induced systemic asphyxia and reoxygenation with the additive effect of the LD cycle, or are they mobilized by the factors oscillating in the circadian dependence, with the additive effect of hypoventilation/reoxygenation?

4. Chronobiological aspects of preconditioning by systemic asphyxia

4.1 Ventricular arrhythmia threshold - A measure of the electrical stability of the heart

There is ample evidence that repeating brief periods of myocardial ischemia and reperfusion may provide protection against electrical instability of the heart evoked by subsequent

ischemia/reperfusion injury. This mechanism, known as ischemic preconditioning (IPC), was first suggested by Reimer et al. (1981) and later elaborated on by Murry et al. (1986).
Similar cardioprotective effects, albeit of variable intensity, have been obtained after pre-treatment with repetitive episodes of hypoxia, which may provide clinical benefit over ischemia in that systemic blood flow into critical organs remains stable (Shizukuda et al., 1993). Most of the available information regarding hypoxic preconditioning (HPC) has come from *in vitro* studies on isolated perfused hearts using transient local hypoxia.
Therefore, it is important to know whether HPC with hypoventilation can also reduce experimentally induced ventricular arrhythmias or increase the electrical stability of the heart against the effect of a prolonged subsequent period of hypoventilation and reoxygenation. We hypothesized that 1. If hypoventilation, similar to ischemia, decreases the electrical stability of the heart, HPC with hypoventilation could have an effect comparable to IPC. Moreover, we focused on whether there were differences in the conditions and dynamics of the developing protective effects of myocardial PC applied during the light (nonactive) and dark (active) parts of the rat regime day and aimed to obtain an understanding of the chronophysiological aspects of this phenomenon in *in vivo* rat experiments; 2. If the autonomic nervous system participates in IPC-induced cardioprotection, it would also participate in the process of HPC. If the autonomic nervous system plays a role in HPC, the effect would depend on external periodicity because cardiovascular and autonomic nervous functions show dependence on 24h periodicity. Thus, the design was aimed to examine the effect of the LD cycle adaptation on the VAT, marker of the electrical stability of the heart, and on heart rate responses, as a marker of autonomic drive, during the post-anaesthetic state, hypoventilatory hypoxia and cardiac preconditioning induced by repeated asphyxias *in vivo* (Svorc & Bracokova, 2003; Svorc & Benacka, 2008; Svorc et al, 2011).
The main aim of these studies was to gain information about the chronophysiological aspect of cardioprotection by hypoventilation-induced asphyxia preconditioning in *in vivo* rat experiments. The experiments were performed in anaesthetized (ketamine/xylazine anaesthesia, ketamine 100 mg/kg [Narkamon, Prague] + xylazine 15 mg/kg [Rometar, Prague] i.m) rats (weight, 300 ± 15 g; 3 to 4 months of age). The rats were adaptated to a LD cycle of 12h:12h, with the dark part of day from 06:00h to 18:00h for 4 weeks and they were divided into 4 groups. During the experiments, all animals were subjected to 20 min of artificial hypoventilation-induced asphyxia, followed by a 20 min recovery period (reoxygenation). The first group of animals was not preconditioned (n = 19) and the other three experimental groups were preconditioned by one (1PC group; n = 9), two (2PC group; n = 15), and three (3PC group n = 11) 5 min cycles of hypoventilation (5 min), each separated by 5 min cycles of reoxygenation (Scheme 1).
The chest was opened by parasternal thoracotomy and after gentle mediastinal preparation, the heart was exposed. The VAT was estimated as the minimal amount of electrical current (mA) needed for elicitation of ventricular arrhythmias by direct electrical stimulation of the heart (400 ms series of rectangular pulses; frequency, 30 Hz; and 10 ms impulse lengths). Stimuli were triggered by the onset of the R wave in lead II of the ECG and the current intensity was increased progressively by steps of 0.2 mA until ventricular arrhythmias were obtained. Recovery of the sinus rhythm was spontaneous. Control recordings of VAT were performed after surgical interventions and a 5 min period of artificial ventilation with the parameters of the normal pulmonary ventilation. Values of VAT were measured in the 5^{th}, 10^{th}, 15^{th}, and 20^{th} min of hypoventilation and in the same intervals during ventilatory recovery.



Scheme 1. Protocol of experiments using preconditioning (PC) by systemic asphyxia. The black-white columns refer to the initial phase of experiments with heating of the animals to the rectal temperature measured before the application of the anaesthetic agent, tracheotomy, thoracotomy, 5 min. period of stabilization (normal artificial ventilation at the parameters of the artificial ventilation V_T 1 ml/100 g of body weight and respiratory rate 50 breaths/min.). The hatched columns represent 5 min. cycles of PC by systemic asphyxia. The empty bars represent 5 min. cycles of reoxygenation, while the black columns represent 20 min. cycles of hypoventilation.

The measurement of heart rate (the mean value of the last 4 cycles) was performed in intact animals (before the surgical interventions in the supine position, spontaneous breathing), after tracheotomy and thoracotomy, after each minute of 5 min. stabilization (the parameters of the normal artificial ventilation), after each minute of PC cycles by systemic asphyxia and after each minute of 20 min. hypoventilation. Because the animals from each group passed through the same conditions from the start of the experiment, heart ratee were summed and one average value was calculated for intact animals (Ini), after the tracheotomy (Tr), thoracotomy (To), during the period of stabilization (Stabil) and during the single cycles of asphyxic PC.

Animals were artificially ventilated by humidified room air at the parameters of the initial ventilation and reoxygenation: respiratory rate 40 breaths/min. and tidal volume 1 ml/100g body weight. During experimental hypoventilatory asphyxia, the respiratory rate and tidal volume were reduced to 20 breaths/min and 0.5 ml/100g b.w., respectively. The respiratory effect of the ventilation was monitored by the analysis of the pH, pO_2, pCO_2, and O_2 saturation from blood samples taken from the femoral artery.

The control values of VATs in the experimental groups did not show any significant difference, although systematically higher values were found during the dark part of the day compared to the light part of the day (control light, 1,87 ± 0,80 mA vs. control dark, 2,12 ± 0,93 mA; 1PC light, 1,96 ± 0,73 mA vs. 1PC dark, 2,44 ± 0,68 mA; 2PC light, 2,19 ± 1,21 mA vs. 2PC dark, 2,48 ± 1,20 mA; and 3PC light, 2,32 ± 0,69 mA vs. 3PC dark, 1,85 ± 0,69 mA) (Figure 5).

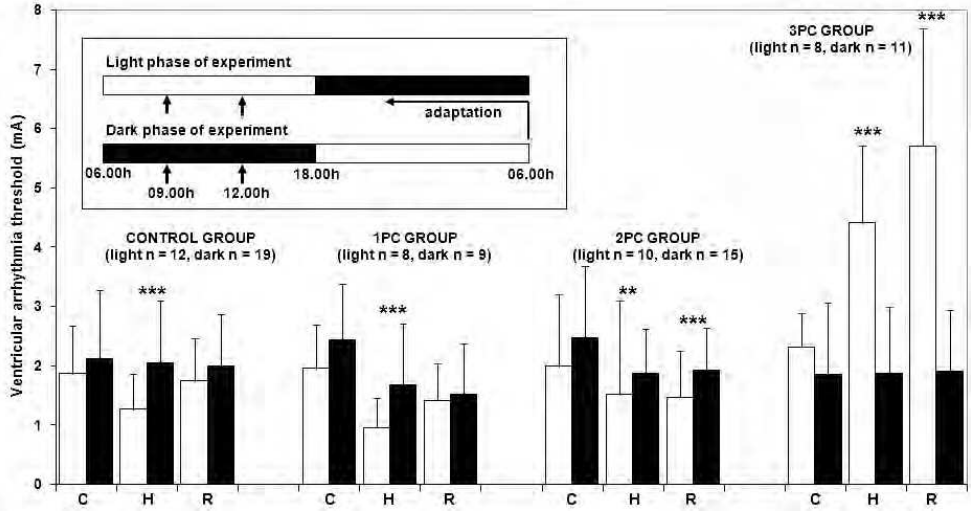


Fig. 5. Mean ± SD values of the ventricular arrhythmia threshold immediately before preconditioning (C), during 20 min. hypoventilatory asphyxia (H) following 20 min. reoxygenation (R) in the control animals (control group) and groups preconditioned by 1 (1PC group), 2 (2PC group) and 3 (3PC group) short cycles of hypoventilation-induced systemic hypoxia, hypercapnia and acidosis. Empty and black columns refer to light and dark parts of the day, respectively. Embedded scheme shows the timing of trials (arrows) in animals adapted to the light-dark cycle. *** $p < 0,001$, ** $p < 0,01$.

During the dark part of the day, hypoventilation non-significantly decreased the VAT in the group without PC (2,12 ± 0,93 mA [control] vs. 2,05 ± 0,85 mA [hypo]); in 1 PC (2,44 ± 0,68 mA [control] vs. 1,68 ± 0,87 mA [hypo]); and in 2 PC (2,48 ± 1,2 mA [control] vs. 1,87 ± 0,60 mA [hypo]). In the 3 PC group, the VAT was not changed and remained at the level of the pre-hypoventilatory value (1,85 ± 0,69 mA [control] vs. 1,87 ± 0,76 mA [hypo]). During the light part of the day, similar but significant VAT decreases were found in the group without PC and in the 1 PC group and non-significant decreases were found in the 2 PC group. In the 3 PC group, where the VAT was markedly increased ($p < 0,001$) above the control value. Significant LD differences were seen in all groups, with higher values in the dark part of the day, except the 3 PC group which had a higher VAT in the light part of the day**.** In the dark part of the day, reoxygenation after one and two cycles of HPC did not change and recovery of the VAT to control values and values from the period of hypoventilation (1 PC group, 2,44 ± 0,68 mA [control] vs. 1,68 ± 0,87 mA [hypo] vs. 1,53 ± 0,58 mA [reoxy]), (2 PC group, 2,48 ± 1,2 mA [control] vs. 1,87 ± 0,60 mA [hypo] vs. 1,93 ± 0,57 mA [reoxy]). In the group without preconditioning (2,12 ± 0,93 mA [control] vs. 2,05 ± 0,85 mA [hypo] vs. 2,00 ± 0,86 mA [reoxy]) and in the 3 PC group (1,85 ± 0,69 mA [control] vs. 1,87 ± 0,76 mA [hypo] vs. 1,91 ± 0,69 mA [reoxy]) the VAT was not changed and remained on the pre- and hypoventilatory levels. In the light part of the day, similar VAT changes were seen in all groups, except the 3 PC group, where the VAT was markedly ($p < 0,001$) increased versus control and hypoventilatory values. The higher VAT values were found in all groups, with higher values in the dark part of the day, except the 3 PC group, in which a higher VAT occurred in the light part of the day. A significant effect of the PC by the HPC was not

confirmed by the χ^2 test nor for hypoventilation ($p < 0,09$) or for reoxygenation ($p < 0,64$) in the dark part of the day. In the light part, such significance was confirmed only for a prolonged period of hypoventilation ($p < 0,001$), but not during reoxygenation ($p < 0,39$).
A considerable intraindividual variability of results is a problem concerning mainly *in vivo* studies, which was also confirmed in our experimental groups. Such variability can be explained by production of spontaneous unpredictible alterations in the electrical stability of the heart induced by anaesthesia or hormonal and homeostatic reflexes operating only in intact animals (Lubbe et al., 1975).
LD differences in the VATs are probably a reflection of the changes in electrophysiological properties of the myocardium. These changes after HPC were also evident in the background of our observations. Possible mechanisms of protection might involve a faster shortening of the action potential (Tan et al., 1993; Ravingerova et al., 1998), also reflected as a shortening of refractoriness (Grover et al., 1994) during hypoxia after PC. Moreover, the duration of arrhythmic activity was significantly shorter in papillary muscles from the hearts of guinea pigs after HPD (Kamasaki et al., 1997), which refers to the fact that HPC can significantly attenuate arrhythmic activity. Unfortunately, these experiments were performed without LD dependence; therefore no information regarding the effect of HPC could be gained. Thus, the question remains whether the effects of these electrophysiological changes protecting the myocardium also depends on the LD cycle. Our results indirectly confirm the fact that the above described electrophysiological changes resulting from PC are probably more effective mainly during the light (nonactive) part of the rat regime day (Svorc et al., 2003).
The effect of PC also depends on the balance between the intensity of the first stimulus and the duration and severity of the prolonged stress. Following the changes in VAT during hypoventilation/reoxygenation, one cycle of HPC had an identical proarrhythmogenic effect in both light parts of the day, but with significantly higher values in the dark part of the day. However, the LD discrepancies in the VAT changes occurred during reoxygenation. In the light part of the day, reoxygenation partly recovered the VAT (antiarrhythmogenic effect), but in the dark part of the day, it was followed by a further decrease in VAT (proarrhythmogenic effect). In both light parts of the day, although hypoventilation/reoxygenation still decreased the VATs in the 2PC group, the decrease was not significant, values were higher than in the 1PC group, but with the preservation of LD differences. Reoxygenation was without effect. The three cycles of HPC stabilized the VAT in the dark part of the day, but a marked and significant cardioprotection against the hypoventilation/reoxygenation decrease of the electrical stability of the heart was detected in the light part, meaning that there are different reactions of the rat myocardium for the**HPC** in the dependence on the LD cycle.
Although the average hypoventilatory VAT value was lower in the 1PC group compared with the hypoventilatory VAT value from the control group (without HPC) in both light parts of the day, the VAT increased gradually in the dependence on the number of cycles of HPC. It appears that 1) one cycle of HPC is too weak of a stimulus for the production of cardioprotection in both light parts of the day; 2) the cardioprotection probably starts after two cycles of HPC in both light parts; and 3) the effect of HPC depends on the numbers of HPC cycles and the LD cycle - it is highlighted by three cycles of HPC.
The dependence of cardioprotection on the number of HPC cycles has been confirmed by others. In isolated rat hearts Testoni et al. (2000) and later Cerruti et al. (2002), showed that as long as the animals were exposed only to hypoxia (60 min.) and reoxygenation (60 min.), without HPC, the more severe atrial and right ventricle contractile disorders and less posthypoxic recovery (other endpoints of PC) were found. Whereas HPC by one 5 min.

cycle of hypoxia and subsequent 10 min. reoxygenation had a small effect, PC with two cycles of hypoxia exacerbated the contractile changes. O'Connor & Merrill (1995) referred to the fact that initial exposure to hypoxia can protect myocardium in *in vivo* conditions against arrhythmias during the second hypoxic period (significant percentage decrease of ectopy incidence). Blockade of cardiac β-adrenoceptors attenuated the incidence of arrhythmia in the second hypoxic period, demonstrating the possible role of catecholamines in the course of HPC. Myocardial ischemia, as well as non-ischemic hypoxia, stimulate efferent adrenergic nervous endings (Daly & Scott, 1963, 1964; Herrmann & Feigl, 1992), the assumption being that the ventricular arrhythmias induced by systemic hypoxia depend on intact adrenergic innervation (O'Connor & Merrill, 1993), which was also shown in our experiments. These interventions deliver possible protection by PC against electrogenic and mechanical effects of the prolonged ischemic period of the myocardium (Lasely et al., 1993). The differences in the number of cycles of hypoxia necessary for the mobilization of the cardioprotective mechanism in the prezent study and previous studies performed *in vitro* and *in vivo*, could be explained by different experimental procedures. Low-oxygen perfusion of isolated hearts *in vitro* (Testoni et al., 2000; Cerruti et al., 2002) may facilitate cardioprotection much sooner compared to an *in vivo* condition. The anaesthesia in *in vivo* experiments is an important variable, as is the animal species in use, e.g., ketamine anaesthesia inhibits PC with anoxia in rats (Ko et al., 1997), in rabbits (Han et al., 2002), and in our results, or α-chloralose in beagles (O'Connor & Merrill, 1993).

4.2 Heart rate – A measure of autonomic nervous system activity

Cyclic fluctuations based on subdiurnal, circadian or supradian cardiovascular responses are also influenced by the short- and long-term variability of the autonomic nervous system. Previous data showed that daily rhythmicity in sympathetic and parasympathetic nerve tone in healthy organisms is paralleled by corresponding changes in the electrophysiological properties of the myocardium (Cinca et al., 1986). Circadian variability in the autonomic nervous system might also represent a substantial influence on the electrical stability of the myocardium under pathological conditions including systemic hypoxia, pulmonary hypoventilation, asphyxia and acidosis (Meurling et al., 2001; Simantirakis et al., 2001; Watanabe et al., 2002).

It is known that both the hypoxic changes in the phasic and tonic drive od the autonomic nervous system and the alterations in the sensitivity of the myocardium to autonomic nervous drive, may also be involved in the effect of PC. It is now apparent that protection from IPC spreads from distant organs to the heart (Pell et al., 1998; Wolfrum et al., 2002) possibly via activation of the autonomic nervous system (Gho et al., 1996; Schoemaker & Van Heijningen, 2000; Liem et al., 2002; Wolfrum et al., 2002). It is possible that the release of local triggers of IPC activates the autonomic nervous system either directly (Schoemaker & Van Heijningen, 2000; Liem et al., 2002) or via sensory nerves (Tang et al., 1999; Xiao et al., 2001; Hu et al., 2002), and transfers the signal to the myocardium or other remote tissues.

Evidence exists that sympathovagal regulation might be related to the protective mechanism of IPC (Loukogeorgakis et al., 2005; Wu et al., 2005). IPC is mediated by sympathetic neurotransmitter release and α_1-adrenergic receptor stimulation (Banerjee et al., 1993; Cohen et al., 2001). Acetylcholine, the parasympathetic mediator, is also involved in the IPC triggering process (Cohen et al., 2001). The anti-arrhythmic protection afforded by IPC may be mediated by preservation of autonomic function (Miyazaki & Zipes, 1989). Other evidence implies that IPC may affect sympathovagal activity from the initial to the target effect (Airaksinen et al.,

1995; Pasceri et al., 1996). Brief coronary occlusion may result in severe autonomic reaction as measured by reduced heart-rate variability; however, the autonomic reaction after further coronary occlusion has been significantly smaller (Woo et al., 1994; Airaksinen al., 1995; Huikuri & Makikallio, 2001). These phenomena highlight the importance of cardiac autonomic regulation in the IPC protective process.

The above mentioned results clearly refer to probable autonomic nervous system participation in cardioprotection induced by IPC. Although HPC is less studied, it is known that pre-treatment with repetitive episodes of systemic hypoxia or hypoventilation-induced asphyxia under *in vivo* conditions evoked not only the similar cardioprotective effects (Shizukuda et al., 1993) but also showed marked LD dependence (Svorc et al., 2003). In the present, the date about the autonomic nervous system participation in HPC are absent and especially, in the dependence on the environment periodicities.

Results of a study with HPC show that the initial heart rate data measured in the intact spontaneously breathing ketamine/xylazine-anaesthetized animals (Ini) treated during the light part of the day (LP) were significantly lower compared to those from the dark part of the day (DP) (M ± SD, 231 ± 28 vs. 264 ± 31 beats/min. $p < 0{,}001$). Similar LD-dependent differences in the averaged heart rate values were maintained after tracheotomy, thoracotomy (To; LP 168 ± 39 vs. DP 218 ± 57 beats/min. $p< 0.001$) and after the onset of the artificial pulmonary ventilation (5 min after the onset, LP vs. DP, 202 ± 34 vs. 262 ± 44 beats/min $p < 0{,}001$). Interestingly, while the heart rate values in DP-treated animals usually returned to close to initial values within 1-5 min. of artificial ventilation (5 min, 262 ± 44 vs. Ini, 264 ± 31 beats/min,) similar recovery was not seen in LP-treated animals (5. min, 202 ± 34 vs. Ini 231 ± 28 beats/min. (Figure 6).

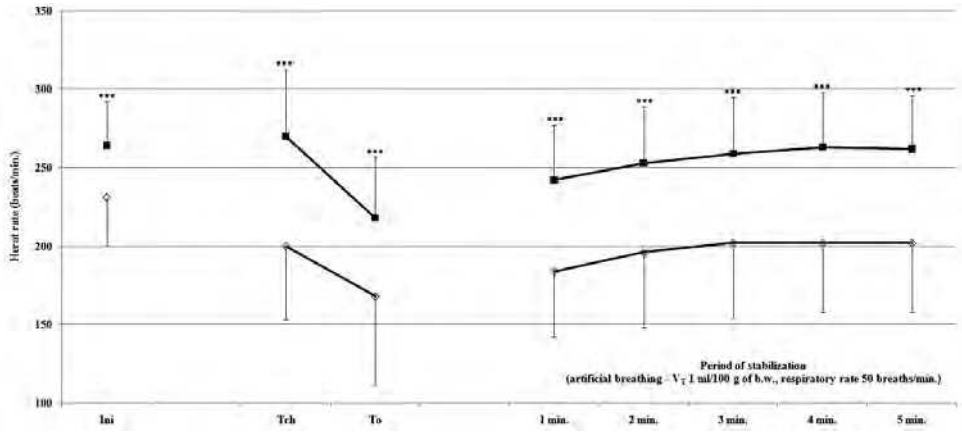


Fig. 6. The average heart rate (HR) values (mean ± SD) before, during and after the surgical interventions in the light (empty rhombus) and the dark (black square) part of the rat regime day. Ini – animals before the surgical interventions in ketamine/xylazine anaesthesia, spontaneous breathing), Tch – immediately after tracheotomy, To – immediately after thoracotomy and after 1., 2., 3., 4. and 5. min. of artificial ventilation (period of stabilization). *** $p < 0{,}001$ statistically significant differences between heart rates measured during the light and dark part of the rat regime day.

Statistically significant LD differences were found in each cycle of PC using hypoventilation-induced systemic asphyxia (Figure 7). HR changes in each cycle of asphyxic PC showed LD dependence. In the light period of the rat regime day, HR was significantly increased in the 5. min. vs. 1. min. in each cycle (1. cycle 203 ± 36 vs. 191 ± 35 beats/min., $p < 0,05$; 2. cycle 208 ± 36 vs. 190 ± 32 beats/min., $p < 0,01$; and 3. cycle 202 ± 34 vs. 189 ± 35 beats/min., $p < 0,01$). In the dark period, the significant differences between 1. min. and the 5. min. of each cycle were not detected (Figure 7).

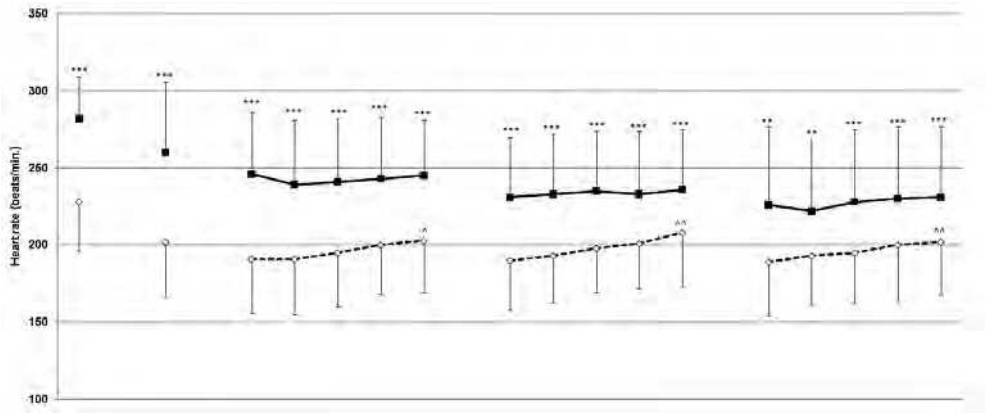


Fig. 7. Average values (mean ± SD) of heart rate during hypoventilation-induced asphyxia preconditioned cycles in the light (empty rhombus) and in the dark (black square) part of the rat regime day. Ini – average heart rate value from the anaesthetized rats before the surgical interventions and at the spontaneous breathing, Stabil – average heart rate value from the 5. min. of artificial ventilation. *** $p < 0,001$; ** $p < 0,01$ statistically significant differences between heart rates measured during the light and dark part of the rat regime day. ^ $p < 0,05$; ^^ $p < 0,01$ statistically significant heart rate difference between the 1. and 5. min. of the preconditioned hypoventilation-induced systemic asphyxia cycles.

In the light part of the day, the heart rate increased gradually with the duration of hypoventilation until the 10. to 11. min. mark in all experimental groups and with the followed stabilization in the control, 1PC and 2PC groups to the end of the asphyxic period. The next heart rate increase was seen only in the 3PC group with the significantly higher values in the 20. min. of hypoventilation against control, 1PC and 2PC groups (3PC vs. control, 235 ± 36 beats/min. vs. 215 ± 36 beats/min., $p < 0,05$; 3PC vs. 1PC, 235 ± 36 beats/min. vs. 196 ± 26 beats/min., $p < 0,01$ and 3PC vs. 2PC 235 ± 36 beats/min. vs. 209 ± 29 beats/min., $p < 0,002$). In the dark part of the day, the heart rate was stabilized in the course of the whole period of asphyxia in all experimental groups (Figure 8).

The spontaneously breathing rats under the ketamine/xylazine anaesthesia are in asphyxic conditions from the start of the experiment *in vivo*, independent of the LD cycle (Svorc et al., 2009). Thus, the disruptive effect of hypoxia on the LD-dependent differences in heart rate response curve was not demostrated, as suggested by the previously mentioned authors. One of the main conclusions from the present study was that heart rates were significantly and systematically higher in the dark part of the regime day than in the light part of the day, and also in asphyxic conditions even if the heart rate response curves in either condition practically paralleled one another.

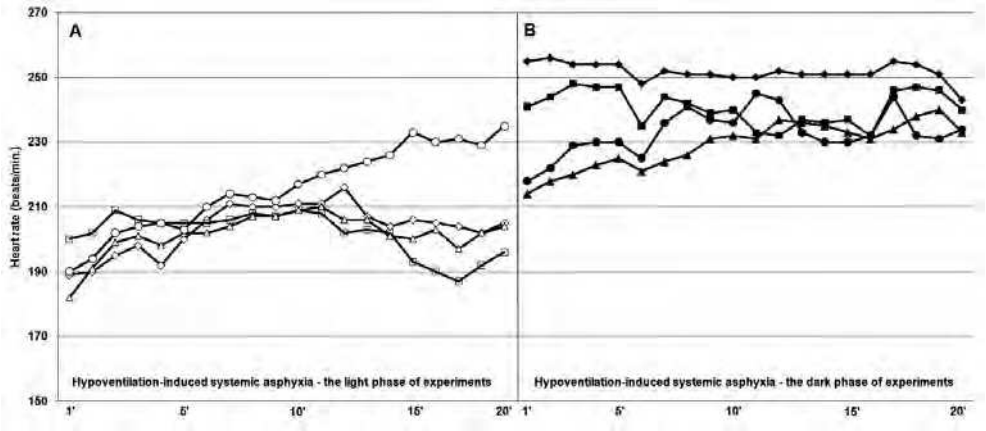


Fig. 8. Mean ± SD values of heart rate during hypoventilation-induced systemic asphyxia in the control group and in the groups pre-treated with a number of different preconditioning-cycles in the light (empty symbols) and the dark (filled symbols) parts of the rat regime day, respectively. Control group – rhombus, 1PC group – square, 2PC group – triangle and 3PC group – circle.

Interestingly, we found that this type of anaesthesia in rats expressively increases parasympathetic tone and decreases sympathetic drive, respectively. The results of heart-rate variability analysis show that in intact spontaneously breathing rats, power HF was significantly higher (light 19,387 ms^2; dark 3,129 ms^2) than power LF (light 0,974 ms^2; dark 0,432 ms^2) and there was also significant LD difference in both parameters ($p < 0.01$ for HF; $p < 0.02$ for LF) (Svorc, Jr. unpublished results). Although heart rates were on the level of bradycardia, the onset of asphyxia was regularly accompanied by a decrease in heart rate, which increased in intensity within a 5 min period in both light and dark parts of the day and persisted for the next 20 min. Atropine-resistant hypoxic bradycardia in rats was also reported by Kaplan et al. (2003) and in isolated ischemic rat hearts by Chanine et al. (1993) who explained that the response was due to a reduction of tissue noradrenaline in the ischemic rat myocardium. In contrast, in rats under moderate hypoxia, Ohkuwa et al. (2005) observed increased plasmatic noradrenaline levels as an indicator of increased sympathetic stimulation. The increase in heart rate was observed only after the first hour in the process of acclimatization the decrease was found with the amplitude reduction of the diurnal variation of heart rate (Kawaguchi et al., 2005). The relative contribution of afferent feedback, autonomic nervous drive and direct hypoxic effect on circulatory responses was examined by Hayashida et al. (1996) in conscious rats with or without chemoreceptor/baroreceptor input. In intact animals, they found that hypoxia facilitated sympathetic activity, while in chemodenervated animals hypoxia induced a decrease in blood pressure, heart rate and renal sympathetic activity. Nevertheless, their data from hypercapnic hypoxia suggest that CO_2–dependent chemical drive may contribute to larger parasympathetic influence to the heart, similar to asphyxia used in present work. In addition to differences in the hypoxic protocols, afferent inputs and behavioural state, sex-dependent differences may play an additional role. Hinojosa-Laborde & Mifflin (2005) reported increases in heart rate after exposure to intermittent hypoxia in males but not in females, in whom the response could even be opposite.

Adaptation in the light part of the day has an important effect on the efficiency of the PC mechanism *in vivo* as shown in our previous studies (Svorc et al., 2003). This later finding increases the practical value for pharmacological interventions, since the majority of the current data on the myocardial IPC or HPC come almost exclusively from in vitro studies. Significant LD cycle effects on heart rate responses, as a marker of autonomic nervous drive, were not reported previously during defined stages of open-surgery preparation.

In conclusion, the study showed that the effect of HPC depends on the LD cycle as well as on the number of PC cycles in *in vivo* conditions in rats. Cardioprotection against the hypoventilation/reoxygenation-induced decrease in the electrical stability of the heart likely begins to occur after two cycles of PC with asphyxia in both light parts of the rat regime day. If stabilization of the electrical stability of the heart is also considered to be a possible means of cardioprotection, then hypoventilation/reoxygenation myocardial injury is minimized only after 3 cycles of PC with asphyxia in the dark (active) part of the day, and the cardioprotection proved to be effective only after 3 cycles of PC by asphyxia in the light (nonactive) part.

This may suggest that while PC with 1-2 short asphyxias does not obviously alter LD-dependent differences in autonomic drive to the heart, several more cycles may eliminate the circadian effects. As to the variability and effects of PC by asphyxias, heart rate responses during the light part of the regime day obviously showed less pronounced dependence on the number of PC cycles and exhibited less interindividual variability than in the dark part of the day particularly during the first half of 20 min hypoventilatory challenge. In the group adapted to the light part of the regime day with 3 PC cycles, heart rate responses after 10 min of recovery lost obvious LD-dependence. Whether observed LD-dependent effects upon asphyxia merely reflect the variations in the autonomic nervous inputs, or represent more complex effects including remodelling of the PC mechanism, alterations in the sensitivity of cardiac conductive system, local effects of hypoxia and acidosis on myocardium remain unclear and require further study.

5. Conclusions

Cardiovascular responses show circadian fluctuations and significant dependence on the LD cycle in pentobarbital- and ketamine/xylazine-paralyzed rats, confirming that LD-related differences are not merely transient or procedure dependent. It is a systematic response assured by distinct neuro-humoral regulation during the light and dark parts of the day, and also under both types of anaesthesia.

This suggests that synchronization to local time may be an important factor in the evaluation of cardiovascular risks in patients also suffering from various respiratory disorders. Analyses of myocardial reactions to acute systemic asphyxia, and to reoxygenation are very important in cardiology because the myocardium reacts differently depending on synchronization to external environmental periodicity.

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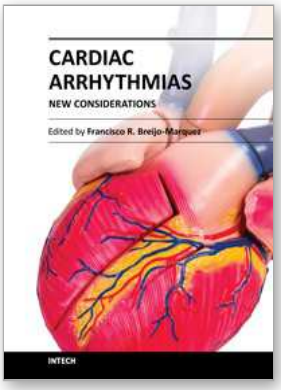

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The most intimate mechanisms of cardiac arrhythmias are still quite unknown to scientists. Genetic studies on ionic alterations, the electrocardiographic features of cardiac rhythm and an arsenal of diagnostic tests have done more in the last five years than in all the history of cardiology. Similarly, therapy to prevent or cure such diseases is growing rapidly day by day. In this book the reader will be able to see with brighter light some of these intimate mechanisms of production, as well as cutting-edge therapies to date. Genetic studies, electrophysiological and electrocardiographyc features, ion channel alterations, heart diseases still unknown , and even the relationship between the psychic sphere and the heart have been exposed in this book. It deserves to be read!

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Phone: +86-21-62489820
Fax: +86-21-62489821