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RESEARCH ARTICLE

RAT ECG AND GENERAL ANESTHESIA: A CHRONOBIOLOGICAL STUDY

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ABSTRACT

This investigation aimed to review the initial state of electrophysiological parameters that may predict the development of heart rhythm disorders in spontaneously-breathing pentobarbital-, ketamine-xylazine- and zoletil-anesthetized rats. These data will contribute to building a wider framework for a chronobiological perspective on the dependence of these parameters on the light-dark (LD) cycle under *in vivo* conditions. The study was performed using female Wistar rats, which were adapted to a 12 h LD cycle. Parameters evaluated included RR, PQ, QT, QTc intervals, the QRS complex and the amplitudes of P, R and T waves. The longest RR and QT interval duration occurred after the administration of ketamine-xylazine anesthesia in both the light period and the dark period. The longest PQ and QTc interval durations occurred under zoletil anesthesia in the light period. It is concluded that from a chronobiological perspective, the most significant electrophysiological myocardial susceptibility toward the potential risk for developing ventricular arrhythmias occurred under ketamine-xylazine anesthesia. The most important predisposition toward the development of ventricular arrhythmias related to disorders of impulse production and conduction was associated with zoletil anesthesia only in the light period. Ventricular arrhythmias resulting from disorders in the dispersion of refractory periods occurred under ketamine-xylazine anesthesia in both the lighted periods.

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INTRODUCTION

Most cardiovascular functions and electrophysiological parameters oscillate over the course of a 24-hour period and exhibit circadian rhythms (Portaluppi and Hermida, 2007; Zhang *et al*., 2014). These functions are influenced by various external stimuli, as well as by endogenous homeostatic mechanisms, resulting in changes in myocardial electrophysiological properties during the day, and enable the cardiovascular system to adapt to rest-activity cycles. There is compelling evidence obtained from animal models and epidemiological studies involving human subjects demonstrating that disruption of circadian rhythms is an important risk factor for several cardiovascular diseases, and that medical intervention may have a time-dependent effect (Muller *et al*., 1985; Culic, 2015; Chen and Yang, 2015). Because heart rhythm disorders can be predicted by alterations in electrocardiographic (ECG) parameters, knowledge of their initial state can significantly affect the interpretation of final experimental results. From this perspective, the development of *in vivo* chronobiological animal models may uncover some of the relationships between circadian rhythms and biological functions, which can be exceedingly difficult to study in human subjects. Performing ethically acceptable cardiovascular research in animals often necessitates the use of general anesthesia. Anesthetic agents used for general anesthesia, however, exert a variety of effects on the cardiovascular system, which may significantly impact cardiomyocytes, conduction systems of the heart, and/or the incidence of arrhythmias (Blake and Korner, 1981, Grund *et al*,. 2004). General anesthesia appears to disrupt cardiovascular stability by altering cardiac function, vascular reactivity and cardiovascular reflexes, which can significantly modify the distribution of cardiac output to selected organs (Akata, 2007). The small number of chronobiological studies that have examined interactions between general anesthesia and circadian rhythms report that general anesthesiahas a significant effect on vital functions (Dispersyn *et al*., 2008). To date, however, there are no published investigations that have evaluated the effect of general anesthesia on basic electrophysiological myocardial parameters and their dependence on circadian rhythms or the light-dark (LD) cycle. Nearly all evaluable ECG parameters are used to identify

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cardiac abnormalities in rats; however, some studies have investigated the reliability of this tool (De Oliveira *et al*., 2012). The time when the experiments were performed and the synchronization of the animals to the LD cycle were not, however, accounted for in the methodologies of these studies. From a chronobiological perspective, this is of concern, because all functions of the cardiovascular system exhibit circadian variations, which makes inter-study comparisons very problematic.

Accordingly, the specific objective of the present *in vivo* study was to assess which type of anesthesia would lead to the most significant electrophysiological myocardial predisposition toward the potential risk for developing ventricular arrhythmias in spontaneously-breathing pentobarbital-, ketamine-xylazine and zoletil-anesthetized rats. This information will contribute to a framework for a chronobiological perspective on the dependence of these parameters on the LD cycle under *in vivo* conditions.

MATERIAL AND METHODS

The study protocol adhered to the Guide for the Care and Use of Laboratory Animals, published by the United States National Institutes of Health (NIH publication number 85-23, revised 1996), and was approved by the Ethics Committee of the Medical Faculty of Safarik University (Kosice, Slovak Republic) (permission number 2/05). The present study was performed using female Wistar rats (mean [±SD] weight 310±20 g, 3 to 4 months of age)after they were adapted to an LD cycle (12h light: 12h dark [intensity of artificial illumination 80 Lux]; *ad libitum* access to food and water; cage temperature 24°C; humidity 40% to 60%; two animals/cage) for 4 weeks. The influence of the light period on the selected parameters was examined after the animals were adapted to an LD cycle (light period from 06:00 h to 18:00 h). The effect of the dark period was monitored after adaptation to the inverse setting of the LD cycle (i.e., light period from 18:00 h to 06:00 h). The animals were divided into three experimental groups according to the anesthetic agent used. Group 1 – pentobarbital intraperitoneal anesthesia (40 mg/kg, Spofa, Prague), light period n = 16, dark period n = 27, group 2 - ketamine (100 mg/kg, Narkamon, Spofa, Prague) + xylazine(15 mg/kg, Rometar, Spofa, Prague) intramuscular anesthesia, light period n = 11, dark period n = 13 and group 3 – zoletil (30 mg/kg, Virbac, France) intraperitoneal anesthesia, light period n = 10, dark period n = 12. Animals were fixed to a preheated table and, after 5 min of rest and spontaneous-breathing in the supine position, ECG data for RR, PQ, QT and QTc intervals, the QRS complex, and the amplitude of P, R, and T waves were recorded and analyzed from bipolar lead II connected to a computer system (ECG Practic Veterinary, Prague). Anesthesia was maintained at a level at which painful stimuli did not evoke conspicuous motor or cardiovascular responses.

Statistical analysis

Data are presented as means ± SD, and were analyzed using GraphPadInStat (GraphPad Software, USA). Analysis of variance (ANOVA) was applied to detect significant differences within a single end-point. The Tukey-Kramer test was used to identify significant differences between groups; $p<0.05$ was considered to be statistically significant. Experiments were performed throughout the course of an entire year, and mean results were calculated independent of season.

RESULTS

RR interval

Unlike pentobarbital-anesthetized rats, a significant LD difference in RR interval duration was found in ketamine-xylazine-anesthetized rats ($p<0.001$) and zoletil-anesthetized rats ($p<0.01$). Rats under ketamine-xylazine anesthesia exhibited the longest RR interval duration in both the light and the dark periods of the regimen day compared with animals under pentobarbital and zoletil anesthesia ($p<0.001$) (Table 1, Figure 1).

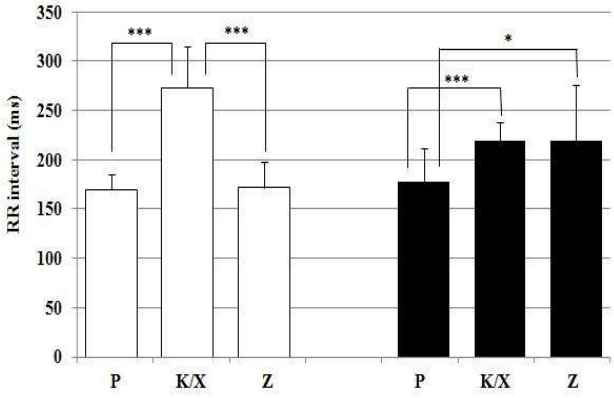

Figure 1. RR interval duration (ms) during light (white columns) and dark(black columns) periods in pentobarbital-(P), ketamine-xylazine-(K/X) and zoletil-(Z)anesthetized rats. Data are presented as means ± SD; *** $p<0.001$ and * $p<0.05$ were considered to be statistically significant differences

PQ interval and amplitude of P wave

No significant LD-dependent differences were found in PQ interval duration in pentobarbital-anesthetized animals compared with the ketamine-xylazine and zoletil groups. Significant ($p<0.001$) differences in PQ interval duration were found between the zoletil and pentobarbital groups, and ketamine-xylazine and zoletil in the light, as well as pentobarbital groups in the dark period (Table 1, Figure 2).
In the light period and the dark period, significant ($p<0.001$) differences in P wave amplitude were found between pentobarbital and ketamine-xylazine anesthesia, and between zoletil and ketamine-xylazine anesthesia. The lowest P wave amplitudes occurred under ketamine-xylazine anesthesia (Table 1, Figure 2).

QRS complex, QT interval, QTc interval and amplitudes of R and T waves

The duration of the QRS complex was not dependent on the LD cycle or on the type of anesthesia, demonstrated by no significant differences between the pentobarbital and the zoletil groups (Table 1). No significant LD differences were found in QT interval duration in the pentobarbital- and ketamine-xylazine-anesthetized animals compared with the zoletil group. In the light period, significant differences ($p<0.01$) in the duration of the QT interval were only observed between the pentobarbital and ketamine-xylazine groups.

Table 1. Electrophysiological myocardial parameters under selected types of anesthesia during the light and dark periods of the rat regimen day

	Pentobarbital		Ketamine/ xylazine		Zoletil	
	Light	Dark	Light	Dark	Light	Dark
RR interval (ms)	169 ± 16	178 ± 34	242 ± 41	220 ± 20***	172 ± 25	219 ± 57**
PQ interval (ms)	44.16±7.7	45.29±4.65	46.82±12.31	36.35±6.97***	51.75±5.43	46.00±3.44***
QT interval (ms)	73.47±15.35	76.02±9.66	84.32±14.94	90.69±7.38	79.36±12.27	70.66±10.37***
QTc interval (ms)	197.7±40.9	190.7±26.6	176.1±25.8	197.5±17***	200.7±28	160.7±33.2***
QRScomplex (ms)	28.47±2.74	28.56±2.56	28,64±2,48	28,89±2,35	28.56±3.54	29.17±3.23
P wave (mA)	0.109±0.04	0.125±0.03*	0.053±0.02	0.059±0.02	0.119±0.03	0.118±0.03
R wave (mA)	0.572±0.18	0.575±0.14	0.593±0.18	0.634±0.12	0.442±0.13	0.631±0.17
T wave (mA)	0.120±0.04	0.123±0.04	0.138±0.03	0.135±0.04	0.126±0.05	0.115±0.03

Data are presented as means ± SD. *** p<0.001; ** p<0.01; * p<0.05 were considered to be statistically significant LD differences.

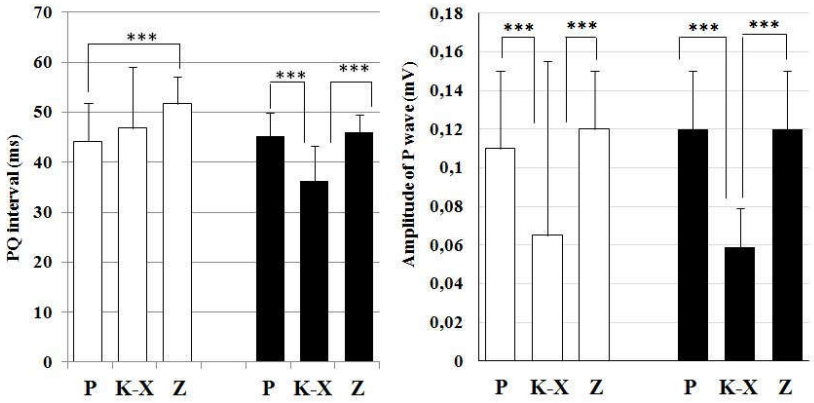

Figure 2. PQ interval duration (ms) and amplitude of P wave (mV) during light (white columns) and dark(black columns) periods in pentobarbital-(P), ketamine-xylazine-(K-X) and zoletil-(Z)anesthetized rats. Data are presented as means ± SD; *** p<0.001; ** p<0.01 and * p<0.05 were considered to be statistically significant differences

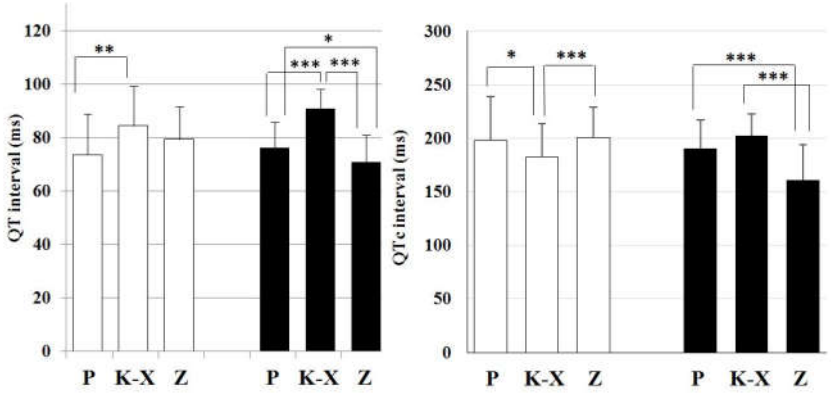

Figure 3. QT and QTc interval duration (ms) during light (white columns) and dark (black columns) periods in pentobarbital-(P), ketamine-xylazine-(K-X) and zoletil-(Z)anesthetized rats. Data are presented as means ± SD. *** p<0.001; ** p<0.01 and * p<0.05 were considered to be statistically significant differences

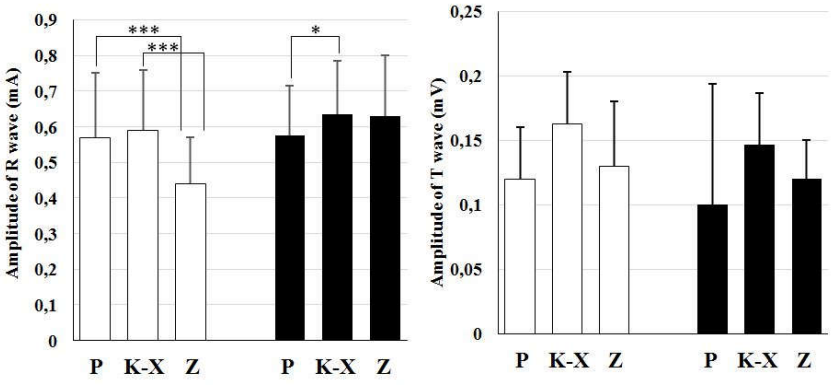

Figure 4. Amplitudes of R and T waves (mV) during light (white columns) and dark (black columns) periods in pentobarbital-(P), ketamine-xylazine-(K-X) and zoletil-(Z) anesthetized rats. Data are presented as means ± SD. *** p<0.001 and * p<0.05 were considered to be statistically significant differences

Significant differences were found between all groups in the dark period of the regimen (Table 1, Figure 3). The shortest duration of QTc interval was under zoletil anesthesia in the dark period, and the longest duration was under pentobarbital anesthesia in the light period. In the light period, significant differences were found between ketamine-xylazine and zoletil anesthesia ($p<0.001$). In the dark period, QTc interval duration was significantly longer under pentobarbital and ketamine-xylazine anesthesia ($p<0.001$) compared with zoletil anesthesia (Table 1, Figure 3). Significantly ($p<0.001$) higher R wave amplitudes occurred under pentobarbital and ketamine-xylazine anesthesia compared with zoletil anesthesia in the light period only. In the dark period, no differences were found. T wave amplitudes exhibited no statistically significant differences between the selected types of anesthesia (Table 1, Figure 4).

DISCUSSION

Analysis of ECG parameters in humans have been shown to exhibit a circadian rhythmicity. P wave duration and area, P-R interval, QRS duration, and QT interval have all demonstrated diurnal changes (Molnar *et al.* 1996, DiLaveris *et al.,* 2001). This circadian variation in cardiac functions, which is dependent on the activity of the autonomic nervous system, is not only exhibited in human subjects, but also in small experimental animals. Therefore, the design and development of *in vivo* myocardial experimental models can be used to demonstrate the presence, or at least possible influence, of circadian rhythms. However, in *in vivo* experiments, general anesthesia must be administered for ethical reasons, which can, to some extent, influence initial measurements and in turn, affect the final results. Therefore, the objective of the present studywas to determine which type of general anesthesia (pentobarbital, ketamine-xylazine and zoletil) would lead to the most significant electrophysiological myocardial predisposition toward the potential risk for developing ventricular arrhythmias in rat models; and to highlight the importance of the initial state of electrophysiological myocardial parameters from a chronobiological perspective.

RR interval

Heart rate (HR) is an easily measurable parameter of heart activity, and can be readily calculated with the duration of RR intervals. Changes in HR may directly impact the cardiovascular system. Caetano and Alves (2015) reported that an elevated resting HR is an independent predictor of cardiovascular and overall mortality in the general population.
HR appears to be strongly affected by the type of anesthesia used. Although Konopelski and Ufnal (2016) refer to HR differences in rats under some types of anesthesia, there is a limitation suggesting that the presented results are probably from the light (inactive) period of the regimen day, or from experiments in which the circadian rhythm of HR was not considered. Regrettably, there are no available published data reporting daytime dependence or synchronization of animals to an LD cycle (i.e., 12 h light: 12 h dark), largely because most *in vivo* experiments involving rats or other similar small animals are usually performed during the non-active (i.e., light) part of the day. In non-anesthetized rats, HR fluctuates as a function of circadian rhythms. Using telemetric studies, Molcan *et al*. (2013, 2014) reported significantly higher HR values during the dark (active) period (355 ± 8 beats/min.; RR interval: 169 ± 7.5 ms) compared with the light (inactive) period of the rat regimen day (315 ± 6 beats/min; RR interval: 190 ± 10 ms). Our results suggest that the effects of selected types of anesthesia on RR interval duration are proportional in both the light and the dark periods of the rat regimen day, given that LD differences were preserved. Based on these findings, assessing the effect of anesthesia on HR, and chronobiologically-dependent electrophysiological myocardial parameters, can be interesting and important. In humans, as well as in larger laboratory animals, increases in HR (shortening of RR intervals) cause a greater predisposition toward ventricular arrhythmias. In small laboratory animals, it appears that the opposite is true. In relation to the electrical stability of the heart in rat models, it has been shown that increasing HR decreases myocardialvulner ability to ventricular arrhythmias (Svorc *et al*., 2003). Decreased HR under ketamine-xylazine anesthesiamay also reflect a greater myocardial predisposition toward ventricular arrhythmia in rats in both the lightperiod and the dark period of the regimen compared with pentobarbitalandzoletil anesthesia. Finally, zoletil anesthesia does not appear to affect cardiovascular function (Tárraga *et al*., 2000; Guarda *et al*., 2007, Musk *et al*., 2014). However, the circadian rhythm of the RR interval (in our experiments presented by the LD cycle) was maintained in all the different types of anesthesia evaluated (Svorc *et al*., 2014; 2015; 2016).

Because LD differences in RR interval duration (or circadian rhythm) are maintained under ketamine-xylazine and zoletil anesthesia (Svorc *et al*., 2014; 2015), these agents likely have a significant influence on K^+, Ca^{2+}, Na^+ channels and the Na^+/K^+ exchanger in pacemaker tissues (Di Francesco, 2006; Bucchi *et al*., 2007), or inhibit sympathetic nervous system activity, which is modulated by the LD cycle, in a similar manner. Based on this finding, our results suggest that prolonged RR intervals may reflect the most significant myocardial predisposition toward ventricular arrhythmia in rats in both the light period and the dark period of the regimen day, mainly under ketamine-xylazineanesthesia.

PQ interval and P wave amplitude

Myocardial susceptibilityto ventricular arrhythmias caused by disorders of impulse production and conduction depends on the velocity of impulse conduction, i.e., on action potential amplitude, which reflects the active role of Na^+ channels (Carmeliet, 1986; Amitzur *et al*., 2000). In pentobarbital-anesthetized rats, we found an LD loss and, based on our results, this may mean that pentobarbital anesthesia significantly affects Na^+ channel dynamics. Na^+ channel dynamics in pentobarbital-anesthetized rats, however, are not directly dependent on alternating light and dark cycles, as they are in ketamine-xylazine-and zoletil-anesthetized rats, although ketamine has been reported to slow ventricular conduction in the rabbit myocardium (Aya *et al*., 1997). In the present study, the longest duration of PQ interval occurred under zoletil anesthesia in both the light period and the dark period of the rat regimen day. The magnitude of the P wave reflects the initial deflection of the cardiac cycle and represents atrial excitation in an electrocardiogram. Atrial excitability in ketamine-xylazine anesthesia was significantly reduced compared with pentobarbital and zoletil anesthesia during both the light and the dark portions of the rat regimen day, which suggests greater susceptibility to heart rhythm disorders.

QRS complex, QT interval, QTc interval, R and T wave amplitudes

It appears that the duration of ventricular depolarization is not affected by specific anesthetics, and is independent of the LD cycle. The wide duration range of QRS complexes (from 12.3 to 57 ms in ketamine-xylazine anesthesia (Regan *et al*.; 2005; Miranda *et al*., 2007; Regan *et al*., 2007) and from 18 to 44 ms in pentobarbital anesthesia (Sugiyama *et al*., 2005; Kumar *et al*., 2009; Ahmad *et al*., 2015)is reported by several authors. The reasoning, therefore, about the increase or decrease in the duration of the QRS complex observed in our experiments may be controversial. If our results are only compared with measurements in the pentobarbital anesthesia group, the QRS complex duration was slightly reduced. Unfortunately, we do not have any comparative values for the QRS complex with regard to ketamine-xylazine and zoletil anesthesia. Prolongation of the QT interval occurs as a result of a defect in ion channels, resulting in an excess of intracellular cations (Viskin, 1999). This occurs due to the leakage of K^+ from cells or excessive Na^+ influx into the cell for the duration of the action potential. Excess intracellular cation concentrations prolong the repolarization of ventricles and cause early afterdepolarization. Prolongation of repolarization further extends the inactivation of Ca^{2+} channels, which also contributes to the formation of early afterdepolarization (Kaluzaj and Pontuch, 2004). The emergence of ventricular arrhythmias resulting from disorders in the dispersion of refractory periods is mainly determined by the duration of QT intervals; QT length therefore appears to be affected by the type of anesthesia. The longest QT interval duration was measured under ketamine-xylazine anesthesia in both the light period and the dark period. This confirms previous results from ketamine studies. Ketamine prolonged the refractory period without changing anisotropy or increasing its dispersion in the rabbit heart (Aya *et al*., 1997), and suppressed norepinephrine-induced inositol trisphosphate (IP_3) production, and inhibition of protein kinase C pathways and a decrease in intracellular Ca^{2+} concentration (Kudoh *et al*., 2002). The duration of the QT interval is mainly influenced by increases in K^+ ion current (Amitzur *et al*., 2000), and it depends primarily on the intracellular concentration of K^+ ions (Froldi *et al*., 1994). Delayed and inwardly rectifying K^+ currents are the primary elements of heart cell electrophysiology that regulate resting potential and repolarization (Baum, 1993; Ko *et al*., 1997; Han *et al*., 2002). Unlike zoletil anesthesia, circadian fluctuations in K^+ currents are probably eliminated under pentobarbital and ketamine-xylazine anesthesia.

Both short and long QTc intervals are associated with an increased risk for disease, and both can lead to death. Ketamine-xylazine anesthesia affected the duration of the QTc interval (in our experiments calculated using Bazett's formula) in an LD cycle-dependent manner. It appears that QTc interval duration is probably independent of the LD cycle under pentobarbital anesthesia. Any prolongation of the QTc interval (pharmacological intervention or other manipulations) are most likely to result in cardiac arrhythmias under ketamine-xylazine anesthesia in the dark (active) period of the rat day. The shortest duration of the QTc interval was observed under zoletil anesthesia, also in the dark (active) period. The amplitudes of the R and T waves are, approximately, a result of an altered state of the myocardium or changed conditions in the body. While the magnitude of the R wave represents early depolarization of the ventricles, our results show that under zoletil anesthesia the rate of depolarization was significantly higher than under pentobarbital and ketamine-xylazine anesthesia, but only in the light portion of the rat regimen day. Because the rats in our experiments did not undergo any procedures, the administration of general anesthesia produced no immediate changes in T wave amplitude, or in the repolarization of the ventricles. Differences in T wave amplitude between the different types of anesthesia, and also LD differences under selected type of anesthesia, were not found.

Conclusion

If prolongation of the RR interval decreases the electrical stability of the rat heart, the most significant myocardial vulnerability to ventricular arrhythmias probably occurs under ketamine-xylazine anesthesia, independent of the alternating light and dark cycles. The most significant predisposition toward the development of ventricular arrhythmias caused by disorders of impulse production and conduction (longest PQ interval) occurred under zoletil anesthesia in the light (non-active) period; ventricular arrhythmias caused by disorders in the dispersion of refractory periods (the longest QT interval) occurred under ketamine-xylazine anesthesia in both the light period and the dark period. From the discussion and conclusions, it is clear that knowledge of the baseline electrophysiological parameters of the healthy heart, as well as their circadian variations, are essential because this information can directly affect the final interpretation of results, especially in *in vivo* experiments in rat models. Findings of the present assert that LD-related differences are not simply transient or procedure dependent, but involve a systemic response initiated by distinct neurohumoral regulation during the light and dark periods of the day and, importantly, also transpire under anesthesia.

Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as conflicts of interest. The author(s) have no financial disclosures.

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