Chapter

The Autonomic Nervous System, Sex Differences, and Chronobiology under General Anesthesia in *In Vivo* Experiments Involving Rats

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Abstract

The aim was to evaluate the current state of the autonomic nervous system (ANS) activity under general anesthesia using heart rate variability (HRV) in dependence on the light-dark (LD) cycle in healthy, sexually mature, spontaneously breathing, zoletil-anesthetized (30 mg/kg) Wistar rats of both sexes after a 4-week adaptation to an LD cycle (12 h:12 h). The animals were divided into four experimental groups according to sex and light period (n = 20 each). RR interval duration, spectral power at very-low-frequency (VLF), low-frequency (LF) and high-frequency (HF), total spectral power of HRV, and the LF/HF ratio were analyzed. Sympathetic and baroreceptor activity was decreased, and parasympathetic activity was increased in both sexes and in both light periods. Regarding sex differences, HRV was significantly lower in females versus males in the light period. In the dark period, females exhibited higher HRV than males. Regarding LD differences, in females, HRV was lower in the light versus the dark period, unlike males, in which HRV was higher in the dark versus the light period of the rat regimen day. Sex differences in the activity of the ANS were apparent in rats, persisted under general anesthesia, and were dependent on the LD cycle.

Keywords: HRV, sex, general anesthesia, chronobiology, rat

1. Introduction

The role of the autonomic nervous system (ANS) and its organ-specific functions have, in large part, been elucidated. Analysis of heart rate variability (HRV) is a popular tool for the assessment of autonomic cardiac control. Small periodic fluctuations in heart rate are well known to physicians and scientific investigators. Because these fluctuations are caused by the varying activity of the ANS, an examination of HRV is needed to obtain information about the functional status of the ANS. Heart rate and changes in heart rate are sensitive indicators of ANS function; therefore, cardiovascular autonomic regulation is considered to be the most reliable indicator of ANS activity.

HRV describes the beat-to-beat variation in heart rate and is used to quantify the interplay between the sympathetic and parasympathetic divisions of the ANS. Although patterns of HRV demonstrate considerable promise for clarifying issues in clinical applications, the inappropriate quantification and interpretation of these patterns may obscure critical issues or relationships, and may impede—rather than foster—the development of clinical applications [1].

HRV analysis, which supports the evaluation of successive RR intervals using electrocardiographic (ECG) methods, has been a powerful tool in the assessment of autonomic cardiac control [2]. For example, in humans, reduced HRV is associated with an increased risk for ventricular arrhythmia and has been shown to be an independent prognostic factor for mortality in patients with cardiac disease(s) [3, 4]. On the other hand, some studies have demonstrated that analysis of HRV spectral performance in rats is an ineffective method for detecting heart-related autonomic control disorders in some experimental models of myocardial infarction or diabetic neuropathy [5–7].

1.1 Chronobiology of HRV

The ANS is an important control system that affects the function of many organs, and its activity is affected by various factors, including age [8], sex, and internal processes, such as circadian rhythm and hormonal fluctuations that slowly rise and fall over the course of 24 h. Circadian fluctuations in HRV parameters in rats were confirmed in a study by Hashimoto et al. [9], who reported that sympathetic nerve activity predominates in the dark phase. The ratio of low frequency (LF) to high frequency (HF) demonstrated a nocturnal pattern, and the value in the dark phase was significantly higher than in the light phase. In 2001, Hashimoto et al. [10] extended the monitoring of circadian rhythmicity in HRV to diabetic rats. Although diabetic autonomic neuropathy modifies circadian rhythms in HRV in diabetic WBN/Kob rats, in healthy nondiabetic Wistar rats, significant light–dark (LD) differences were detected in some of the monitored HRV parameters. In both age-different and pre-diabetic Wistar and diabetic WBN/Kob rats, no LD differences were found in the LF parameter of HRV; however, significant LD differences in the HF parameter were detected, except in older diabetic rats. Significant LD differences were found in the LF/HF ratio, but only in prediabetic Wistar rats.

In a telemetry study, Mamalyga [11] described fluctuations in ANS activity during a 24 h period, in which the control groups of male rats exhibited the greatest predominance of sympathetic activity between 12:00 h and 24:00 h. Similarly, in this time range, the LF parameter and LF/HF ratio exhibited higher values, and the HF parameter of HRV exhibited lower values. Analysis of multiday ECG recordings demonstrated the predominance of different mechanisms of heart rhythm regulation in experimental and control rats over a 24 h period. More severe dysfunction of neuroautonomical mechanisms of regulation in experimental rats was reflected in circadian dynamics. Further evidence supporting the existence of circadian rhythms in ANS activity was obtained in a study by Hsieh et al. [12], who monitored various physiological signals after implantation of sensors into the abdomen of rats and were recorded without interruption for >10 days. There was no difference in sleep/wakefulness patterns, physical activity, body weight, and autonomic functioning assessed according to HRV among control, sham, and experimental rats. Continuous recording further revealed circadian rhythms in HRV parameters, namely a 24 h cycle in RR intervals, the total power of HRV, and HF and LF powers of the RR spectrum. As such, we believe that this information may be useful in future biobehavioral studies.

In common practice, experiments are performed during "regular" working hours, even after the synchronization of rats to the LD cycle (12 h:12 h). Although this synchronization is often described in the methods section of these studies, the time of day when the experiments are performed is not reported. Therefore, it is assumed that the experiments are performed during the day (i.e., during the light) and, thus, on "sleeping" rats in their inactive period of the regimen day. However, the question is, what are the reactions of animals in their active period if there are fluctuations in the functions of individual systems in both sexes? Is there alternative reactivity of these systems, or is there a uniform reaction in both sexes? Therefore, if sex differences in the results of various experimental studies are documented, it is necessary to respect this fact. As such, future studies should decode these questions and try to include females in experiments whenever possible.

In the planning stages and design of *in vivo* experiments, researchers often encounter multiple problems, one of which can be the actual methodology. Established and proven methods are often used and are precisely focused on the type of experiment, whereas other factors that may affect and, consequently, lead to misinterpretation of the results are not taken into account.

1.2 Anesthesia

However, approaches based on ECG recordings of animals in an anesthetic state are not ideal nor valid for HRV analysis due to significant heart rate fluctuations associated with impaired autonomic modulation of the heart [13, 14]. In addition, anesthesia may contribute an important additional risk for animal mortality under some pathological conditions such as myocardial infarction and diabetes mellitus [15–17]. General anesthesia weakens autonomic function and baroreflex control. This side effect should be avoided as much as possible because it limits the ability of the subject to respond to physiological challenges during surgery [18]. Therefore, any research intervention that could affect aspects of the ANS and its impact(s) on internal organs should take into account the anesthetic used. Intravenous anesthetics may have different qualitative and quantitative effects on the peripheral ANS and, thus, may alter the activity of the sympathetic or parasympathetic divisions of the ANS.

1.3 Sex

In the vast majority of experimental studies, only male rats are used; however, there is also the other sex (i.e., female), in which differences may already exist in the very essence of the monitored functional system and exhibit a different response to interventions. At the same time, the study of sex differences is a driving force for development and, in many cases, the basis of health and medicine. However, there are opinions that the investigation of sex differences is ineffectual and does not merit extensive research [19].

Although there are several reasons why female animals are omitted, the primary rationale is simple—males and females are biologically different. Among other reasons, some scientists consider males to be representative of humans and differences from male norms are considered to be atypical or abnormal. Others attempt to "protect" females from the adverse effects of various interventions [20]. Still, others generalize findings from males and females, regardless of differences and, generally speaking, most scientists use male rats because they want to avoid accounting for hormonal cycles in females, which may reduce the homogeneity of the study

population and affect the impact of experimental interventions [21]. When females are included in experiments, two problems arise—the sample size is effectively halved—the economic aspect; and the dispersion of results increases. One explanation for the increased variance is the simple fact that males and females are different and these differences increase the range of variability. However, if males and females are mixed, scientists may find a beneficial effect of a tested drug, for example, that lowers blood pressure, in both sexes [19]. On the other hand, on obtaining results from *in vivo* experiments in rats, errors in general interpretation may arise due to differences related to sex, and these discrepancies occur not only in behavioral studies [22–24]. Furthermore, there are also sex-dependent differences in drug metabolism and the action of liver enzymes [25], in the internal environment [26], in the activity of the ANS and cardiovascular system [27, 28], and most probably, in other functions as well.

As such, whether to acknowledge sex differences in *in vivo* experiments involving rats becomes a legitimate concern. Presently, however, there are relatively little data regarding sex differences in ANS activity or ANS activity during anesthesia. During resting conditions, male rats exhibit a significantly higher heart rate and lower HRV parameters than female rats. This occurs not only during the active but also during the inactive phase of the daily cycle of rats [27]. Further *in vivo* rat studies have confirmed results reported by Koresh et al. [27]—that there are significant sex differences in HRV and depend on the LD cycle, even under conditions of general anesthesia [28]. LD differences with nonsignificantly lower HRV were found in females in the light part compared to the dark part of the regimen day, in contrast to males, in which HRV was significantly higher in the light part of the day.

These data support the concept that sex-based variations should also be taken into account, given that females in human and animal studies exhibit different mechanisms of cardiovascular regulation [29]. Although these data suggest that if there are sex differences in individual cardiovascular parameters, they are predominantly regulated by the ANS. Logically, therefore, if sex differences exist in cardiovascular activities, sex differences in the circadian oscillations of individual divisions of the ANS must also exist in parallel.

The aim of the present study was not to downplay or critique the excellent and valid results of experimental *in vivo* studies involving rats but to raise awareness to the possibility of improving the design of the experiments themselves, not only by respecting sex differences, but also chronobiological principles. Accordingly, the primary goal of this investigation was to determine whether there are sex differences in ANS activity, measured according to HRV dependence on the LD cycle (a parallel to the circadian rhythm) in healthy, sexually mature, spontaneously breathing, zoletil-anesthetized rats.

2. Materials and methods

2.1 Ethics approval

The present study conformed to the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (publication number 85–23, revised 1996). The study protocol was approved by the Ethics Committee of the Medical Faculty of Safarik University (Kosice, Slovak Republic; permission number 2/05 and permission number ŠVPS SR: Ro4234/15–221).

2.2 Animals

The experiments were performed using Wistar albino rats (weight, 340 ± 40 g, 3–4 months of age) acquired from a breeding and vendor company (VELAZ, Koleč, Czech Republic, certificate number 70029/2013-MZE-17214), with veterinary registration number CZ 21760118.

2.3 Adaptation

The animals were quarantined for 2 weeks in the Laboratory of Research Biomodels of the Medical Faculty of Safarik's University in Košice (official number SK UCH 08018) and adapted to an LD cycle (12 h light:12 h dark [intensity of constant artificial illumination during the light period, 80 Lux]); 40–60% humidity; cage temperature 24°C; two animals/plastic cage for 4 weeks. The rats were fed a standard pellet diet, with *ad libitum* access to food and water. Animal handling was performed by the professional staff of the animal facility.

2.4 Anesthesia

Anesthesia (zoletil, 30 mg/kg, Virbac, France) was administered in prescribed doses in the adaptation room by intraperitoneal injection based on the weight of the animal. After testing the effect of anesthesia (loss of uprighting reflexes, reaction to painful stimulus), the animals were transferred to the operating room, where they were fixed to an experimental table on which subcutaneous electrodes were used to record ECG and HRV. Again, the depth of anesthesia was assessed depending on whether the painful stimulus caused noticeable motor movements (minimal limb movement and muscle tension change) or cardiovascular responses such as changes in heart rate or onset of heart rhythm disorders.

2.5 Experimental groups

The effect of the light period on the monitored parameters was examined after adaptation to an LD cycle, with the 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., with the light period from 18:00 h to 06:00 h). The animals were randomly divided into four experimental groups (n = 20 each) according to sex and light conditions—group 1, female (light period); group 2, female (dark period); group 3, male (light period); and group 4, male (dark period). In *in vivo* experiments, at least 20 animals are valid sample size for statistical processing.

2.6 Protocol

HRV was analyzed using the ID Instruments computer system for biopotential recording from an average of 220 heart cycles, 20 minutes after administration of anesthesia at 09:00 h—12:00 h using separate animals. In analyzing HRV parameters, the focus was on the evaluation of RR interval duration spectral power at very-low-frequency (VLF, 0.003–0.04 Hz), low-frequency (LF, 0.04–0.15 Hz), and high-frequency (HF, 0.15–0.4 Hz), total spectral power of HRV, and the LF/HF ratio. The experiments were performed throughout the year and the results were averaged independently of the season and, in females, independently of the estral cycle. All animals

(i.e., 20/20) were included in the statistical analysis. Before and after administration of the anesthetic, as well as during measurement, there were no adverse events or unexpected changes in HRV or ECG parameters, although considerable variability was observed. On completion of the measurement, the animals were transferred to the animal facility.

2.7 Statistical analysis

Data are expressed as mean ± standard deviation (SD). Data were analyzed using InStat (GraphPad, San Diego, CA, USA). The Tukey–Kramer test was used to compare data from the groups, and differences with $p < 0.05$ were considered to be statistically significant.

3. Results and discussion

3.1 RR interval

Evaluation of the RR interval can sometimes be problematic because different effect(s) of the anesthetic on this parameter has been described. The data reported in **Table 1** indicate values of the duration of the RR interval from telemetry studies and under different types of general anesthesia according to sex and dependence on the LD cycle (**Figure 1**).

Baseline RR interval analysis from telemetry studies [9, 7, 27, 30, 31] involving male Wistar rats, in which a chronobiological approach was applied, indicates that there is a circadian rhythm in the duration of RR intervals in rats, with a lower RR interval duration during the active (i.e., dark) period of the regimen day. Although adaptation of animals to the LD cycle was described in these articles, exactly what time of day the measurements were performed was not reported, nor whether they were average values from the entire 24 h period or only from certain time intervals the measurements were performed and recorded. The averaged results of baseline RR interval duration indicate that sex differences are exhibited in both the light and dark period of the rat regimen day; however, more experimental studies are needed to confirm this conclusion.

When comparing the duration of the RR interval in male rats from telemetry studies, it is clear that the shorter duration occurred during the dark period, which corresponds to a higher heart rate. Under zoletil anesthesia, a shorter RR interval was found in both light phases of the rat regimen day compared with values from telemetry studies, indicating a tachycardic effect of this anesthetic. The shortened duration of the RR interval corresponded to increased heart rate in both sexes in both lighted periods of the regimen day. Among females, LD differences were not observed in the duration of the RR interval (light, 142.30 ± 25.19 ms vs. dark, 134.97 ± 9.09 ms), in contrast to males, in which a significantly ($p < 0.001$) longer RR interval was recorded during the light part of the day (light, 145.05 ± 8.51 ms vs. dark, 124.68 ± 5.14 ms). Sex differences were found only in the dark (i.e., active) part of the day, with significantly shorter RR intervals in males. On the other hand, significant LD differences were maintained in males but eliminated in females. Addtitionally, compared to values reported in telemetry studies (**Table 1**), the RR interval was shorter, indicating a higher heart rate.

Our results, therefore, indicate that in zoletil-anesthetized rats, LD differences were maintained only in males but not in females. Considering the results of telemetry studies by Molcan et al. [50, 51], heart rate exhibits a significant circadian

	Light period		Dark period	
Anesthesia	**Female**	**Male**	**Female**	**Male**
Telemetry studies	168.7 (167.3–170.1) (n = 1)	163.2 (157–168.5) (n = 2)	140.2 (139.5–141) (n = 1)	145.9 (142.6–149.2) (n = 2)
Pentobarbital	177 (174–180) (n = 1)	—	165 (163–167) (n = 1)	—
Ketamine	271.1 (231.5–310.7) (n = 2)	—	213.1 (194.1–232.1) (n = 2)	—
Tribromoethanol	—	—	—	—
Thiopental	—	—	—	—
Urethane	—	—	—	—
Zoletil (Present study)	142.30 (117.1–167.5)	145.05 (136.5–153.6)	134.97 (125.9–144.1)	124.68 (119.5–129.8)

Data presented as the average RR interval duration (ms) (range); (n, number of experiments from which RR interval was evaluated).

Table 1.
Duration of RR interval from telemetry studies and for different types of general anesthesia according to sex and dependence on the light–dark cycle.

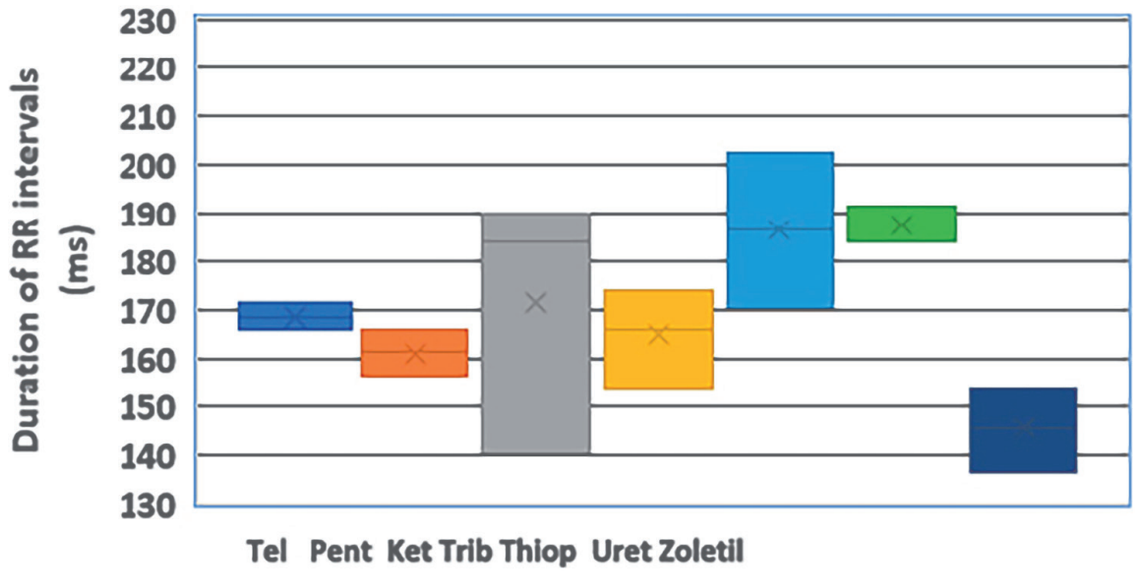

Figure 1.
Distribution of average values and ranges of RR intervals from telemetry studies and under different types of general anesthesia in male rats, without specification of synchronization to the light–dark cycle or the time of day when the experiments were performed. Tel – Telemetry studies (168.5(165.6–171.5), n = 3) [7, 30, 31]; pent – Pentobarbital (161.1(156.1–165.7), n = 6) [32–37]; Ket – Ketamine (183.8(140.2–189.8), n = 5) [38–42]; Trib – Tribromoethanol (166(154–174), n = 1) [43]; Thitop – Tiopenthal (186.2(170.3–202.1), n = 4) [44–47]; Uret – Urethane (187.5(183.7–191.2), n = 2) [48, 49]; zoletil – Zoletil anesthesia (145.05(136.5–153.6), n = 1) [presented results]. Data presented as average RR interval duration (ms) (range); (n, number of experiments from which RR interval was evaluated).

rhythm in non-anesthetized rats, in which the heart rate in the dark period fluctuated from 347 beats/min to 363 beats/min, and from 309 beats/min to 321 beats/min in the light period. Thus, it appears that although zoletil exerts a tachycardic effect,

it can eliminate——or, at least modify——the circadian rhythm of heart rate, but only in females.

Such elimination or modification of LD differences in heart rate among females may also be partly explained by the greater sensitivity of females to acidosis, hypoxia, and hypercapnia under general anesthesia [52]. Previous studies have described the effect of hypoxia on the modulation of daily rhythmicity [53–57]. The fact that hypoxia modifies circadian oscillations of important variables, such as body temperature and metabolism, can lead to the expectation that the rhythms of many functions are interrupted by hypoxia on the basis of their relationship with the primary variables. Such a relationship likely contributes to a greater parasympathetic effect(s) on the heart [58]. Additionally, the effect of anesthetics can contribute to the loss or modification of rhythmicity. For example, in female rats under pentobarbital anesthesia, parasympathetic activity increases and sympathetic and baroreflex activity decreases; however, LD differences in heart rate are eliminated. Under ketamine/xylazine anesthesia, a preference toward parasympathetic activity was increased and sympathetic and baroreflex activity was depressed, resulting in significant bradycardia but with the maintenance of LD differences [59].

The paradox, under ketamine/xylazine anesthesia, therefore, remains—on the one hand, there is clearly evident increased parasympathetic activity and, on the other hand, increased heart rate. This paradox has been described by several authors [60–65], who assumed that stimulation of the vagal nerve releases catecholamines, which in turn can affect heart activity. This is also probably the case with zoletil anesthesia, which may have a similar effect on the release of catecholamines through higher parasympathetic activity, and is particularly evident in males in both light periods of the regimen day. Because sympathetic tone is significantly reduced and parasympathetic tone dominates, it is assumed that the duration of RR intervals is predominantly determined by the parasympathetic system.

3.2 HRV analysis

Despite the large variation in HRV spectral powers under zoletil anesthesia, in terms of sex differences, parasympathetic activity dominated in both sexes and in both light periods. In terms of sex differences, female HRV was significantly lower compared to males in the light period, while in the dark part of the regimen day, it was, in contrast, significantly higher in females compared to males (**Figure 2**).

Sympathetic activity dominates the normal life cycle of rats [7, 65] and zoletil anesthesia increases parasympathetic activity in both sexes. Similar results have been reported in previous studies. Administration of the anesthetic agent tribromoethanol in male Wistar rats [66], ketamine hydrochloride and diazepam in albino Wistar rats [67], and ketamine/xylazine and pentobarbital in females [59] resulted in predominant parasympathetic activity. However, our results indicate that precisely defining changes in HRV are difficult due to significant variability, which in turn makes it difficult to attribute sex differences. Thus, we agree with the opinion described in the introduction that approaches based on ECG recording under general anesthesia are not fully valid for HRV analysis.

In males under zoletil anesthesia—spectral power of HF (parasympathetic activity, r = 0.96) during the light and dark periods of the regimen day, spectral power of LF (baroreflex activity, r = 0.95), but also spectral power of HF (parasympathetic

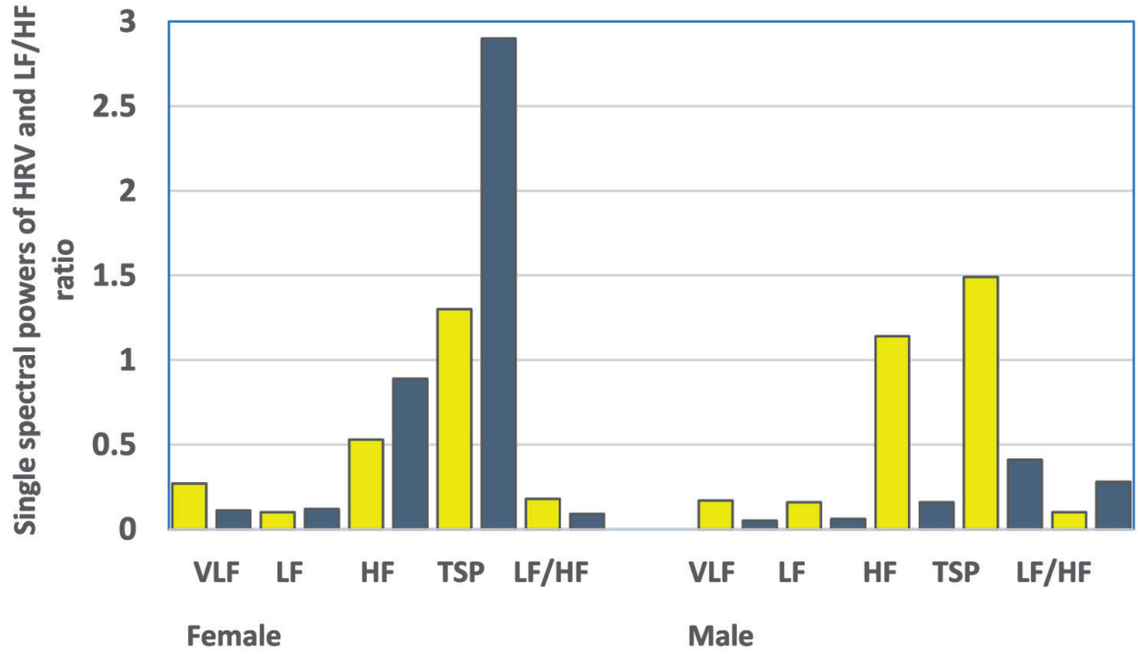

Figure 2.
Representation of heart rate variability (HRV) spectral powers in a rat model under zoletil anesthesia in both sexes. VLF – Spectral power of the very low frequency of HRV; LF - spectral power of the low frequency of HRV; HF - spectral power of high frequency of HRV; TSP – Total spectral power of HRV. Yellow columns – Light period of the rat regimen day; blue columns – Dark period of the rat regimen day.

activity, r = 0.81) significantly contributed to changes in the total spectral power of HRV. Sympathetic activity is practically not involved in the formation of the total spectral power of HRV. The participation of individual spectral powers, as well as the total spectral power of HRV in the duration of RR intervals, is minimal in both lighted periods of the rat regimen day (**Table 2**). After analysis of the dependence

Variable	Sex, light cycle			
	Female, light	Female, dark	Male, light	Male, dark
RR-VLF	**r = 0.54**	**r = 0.58**	r = 0.34	r = −0.13
RR-LF	**r = 0.47**	**r = 0.59**	r = 0.26	r = −0.12
RR-HF	r = 0.37	**r = 0.60**	r = 0.20	r = 0.06
RR-TSP	**r = 0.51**	**r = 0.61**	r = 0.28	r = 0.06
TSP-VLF	**r = 0.6**	**r = 0.87**	**r = 0.59**	r = 0.05
TSP-LF	**r = 0.99**	**r = 0.99**	**r = 0.59**	**r = 0.95**
TSP-HF	**r = 0.92**	**r = 0.89**	**r = 0.96**	**r = 0.81**

Bolded values indicate statistically significant dependence between single parameters. VLF – spectral power of the very low frequency of HRV; LF - spectral power of the low frequency of HRV; HF - spectral power of the high frequency of HRV; TSP – total spectral power of HRV.

Table 2.
Correlation coefficients of RR interval duration between spectral powers of heart rate variability (HRV) and the share of individual spectral powers in changes in the total spectral power of HRV.

of the duration of RR intervals on the total spectral power of HRV, we came to the conclusion that the duration of RR intervals (i.e., heart rate) is not regulated by the ANS in both light periods of the rat regimen day. We assume that other mechanisms are likely involved in the regulation of heart rate and are activated by zoletil.

In female rats under zoletil anesthesia, the spectral power of LF (baroreflex activity, r = 0.99) and spectral power of HF (parasympathetic activity, r = 0.92) contributed significantly to changes in the total spectral power of HRV during the light period of the day and during the dark period proportionally in all three spectral powers of HRV. Sympathetic activity in both lighted periods was involved in the formation of the total spectral power of HRV in females (**Table 2**). After analysis of the dependence of the duration of RR intervals on the total spectral power of HRV, we found that the duration of RR intervals (i.e., heart rate) was under the regulatory influence of the ANS in both lighted periods of the rat regimen day (light, r = 0.51; dark, r = 0.61) with proportional representation of all three spectral powers of HRV.

We conclude that there are sex differences in the total spectral power of HRV in zoletil-anesthetized Wistar rats. In the light period in females, HRV was significantly lower than in males, and vice versa in males in the dark period of the regimen day. This means that, in females, the myocardium may be more sensitive to ANS regulatory interventions in the dark versus the light period. It is generally accepted that decreased HRV is a predictor of myocardial infarction mortality and increased HRV is associated with decreased morbidity and mortality. From this point of view, in zoletil-anesthetized female Wistar rats, during the active (dark) period, there is greater electrical stability in the myocardium than during the inactive (light) period. On the contrary, in males, the heart more sensitive reacts to changes in ANS activity in the light versus the dark period of the regimen day.

In females, changes in HRV were the result of sympathetic (i.e., VLF) and baroreflex (i.e., LF) activities and, in males, parasympathetic (i.e., HF) activity dominated. Among females, changes in RR were primarily due to changes in HRV, whereas in males, changes in HRV had no effect on RR in both lighted parts of rat regimen day. The results of these studies show that not only sex—but also the time of day experiments are performed—also plays an important role [68]. However, supportive evidence of HRV changes in rats during a 24 h period is lacking.

The LF/HF ratio can be used to quantify the changing relationship between sympathetic and parasympathetic nerve activity (i.e., sympathetic-vagal balance) [69–71]. The exact interpretation of the LF/HF ratio also depends on the assumption that physiological interventions always cause mutual changes in parasympathetic and sympathetic activity.

Our results demonstrate that the LF/HF ratio depends on the light periods of the regimen day. In females in the light period, the LF/HF ratio was significantly higher and in the dark period, significantly lower than in males. These conclusions, however, should be interpreted with caution. In a study addressing the meaning of HRV examination, Billman [72] questioned the evaluation of the LF/HF ratio. The LF component of HRV does not provide a cardiac sympathetic response index, but rather reflects a complex and not a readily recognizable mixture of sympathetic, parasympathetic, and other unidentified factors with parasympathetic factors, which account for the largest part of the variability in this frequency range. As a result, it is difficult to recognize the physiological basis for LF/HF. In addition, a relatively large amount of data suggests that the spectral power of the HF component cannot be attributed solely to changes in cardiac vagal efferentation, further compromising the accurate interpretation of the LF/HF ratio [72].

4. Conclusions

In *in vivo* experiments, homeostatic regulatory mechanisms are not eliminated. This means that experimental results are a reflection of a direct but significantly intravariable response of the animals to the administration of anesthetic. On the evaluation of HRV in *in vivo* conditions, replacement and reduction of animals are not possible; however, knowledge about sex differences during anesthesia in the dependence on LD cycle in ANS activity may improve the quality of experimental design. There is little to no data regarding sex differences, and we do not have any data regarding changes in ANS activity depending on the LD cycle under general anesthesia. Further research is needed to assess the responses of other species because the effect of zoletil is essentially not described in experimental practices.

Based on our results, we conclude that under zoletil anesthesia, sympathetic (VLF) and baroreceptor (LF) activity were decreased, and parasympathetic (HF) activity was increased in both sexes and in both light periods. LD differences were preserved mainly in the HF component; thus, the circadian rhythm in parasympathetic activity likely also exists in both sexes. In terms of sex differences based on the total spectral power of HRV, our results suggest that HRV, in the light period of the rat regimen day, was significantly lower in females versus males. In the dark period, females exhibited higher HRV than males. In terms of LD differences, in females, HRV was lower in the light versus the dark period, unlike males, in which HRV was higher in the dark versus the light period of the rat regimen day.

Conflict of interest

The authors declare no conflict of interest.

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