Aim of the study: Melatonin (MLT) is reported to exert uroprotective effect due to its antioxidant/anti-inflammatory properties. It is unknown whether that effect also results from melatonin receptor activation, or it is attributed to the modulation of the autonomic nervous system (ANS) activity. Our purpose was to evaluate the effect of MLT and agomelatine (AMT) – melatonin receptor agonist on ANS activity, indirectly assessed by heart rate variability (HRV), in rats with cyclophosphamide-induced hemorrhagic cystitis (CP-HC).

Material and methods: CP-HC was induced in all rats by four doses of cyclophosphamide given intraperitoneally (i.p.) at the dose of 75 mg/kg/dose. Rats were divided on three experimental groups and during induction of cystitis were treated i.p. with: (1) saline (control group); (2A/2B) MTL given at the dose of 40 or 100 mg/kg/dose; (3A/3B) AMT given at the dose of 40 or 100 mg/kg/dose. HRV recordings were performed in anesthetized rats at the eighth day of the study.

Results: Both 2A and 2B animals were characterized by an increase in all non-normalized components in HRV spectrum. Furthermore, normalized LF (nLF) increase along with normalized HF (nHF) decrease were demonstrated in 2B rats. AMT treatment resulted only in an increase in total power (TP) and very low frequency (VLF) in 3A animals.

Conclusions: CP-HC rats treated with MLT were characterized by global ANS activity elevation, with a marked sympathetic tone predominance in subgroup 2B. Since the AMT treatment had no effect on autonomic function, it seems that melatonin modulates autonomic activity via non-receptor mechanisms.

Key words: cyclophosphamide, hemorrhagic cystitis, autonomic nervous system, heart rate variability, melatonin, agomelatine.
oxide inhibition [8]. Melatonin could also alleviate urinary frequency by suppressing the brain micturition centre [9].

However, the pharmacodynamic description of MLT is still incomplete, and it is possible that melatonin is also an agent targeting the autonomic nervous system (ANS), as an additional pharmacological action. Hence, we aimed to determine whether melatonin modulates the ANS function in an experimental CP-HC model, and whether potential changes in autonomic regulation may contribute to its potential uroprotective properties. Moreover, our goal was also to assess the ANS response to agomelatine (AMT), an agonist of MT1/MT2 melatonin receptors with additional antagonistic impact in relation to the receptors 5HT2C, but without additional melatonin-like antioxidant/anti-inflammatory properties [10, 11]. Using AMT, we tried to clarify either whether the potential neuromodulating effect of MLT may result from MT receptor activation, or if it is attributed to its additional non-receptor pharmacodynamic mechanisms.

**Material and methods**

The medical experiment, which has been described in this paper, was approved by the First Local Ethical Committee for Animal Experiments in Krakow. The experiment was carried out using thirty 11-week-old albino Wistar rats, divided into three groups, ten individuals each. Within 7 days, an experimental cyclophosphamide-induced hemorrhagic cystitis (CP-HC) was evoked in all studied animals, as described previously both Dinis et al. [12] and Chopra et al. [13]. CP-HC developed after four-time (in the first, third, fifth and seventh day kg b.w., respectively) [15–17]. Hence, we adopted 40 mg/kg b.w. MLT/AMT as an “average” dose and 100 mg/kg b.w. as a “high” one. Moreover, we also used the same MLT/AMT high dose in our previous study aimed at assessing the MLT/AMT influence on bladder motility in an experimental model of CP-HC [14]. Melatonin was obtained in crystalline form from Sigma Aldrich, and agomelatine from the commercial preparation Valdoxan®, Servier (tablets containing 25 mg AMT). Both MLT and AMT, after preparation of the corresponding doses, were dissolved in water for injection, immediately prior to administration. On the eighth day of the experiment all of the studied animals were subjected to ECG recording, with subsequent analysis of heart rate variability (HRV). The ECG was recorded under general anaesthesia after urethane administration (1200 mg/kg, i.p.) and 20-min rest. Before recordings, the abdominal fur was removed, and the abrasive paste and standard ECG gel were applied. Recordings were collected with the paediatric Ag/AgCl use (EG-S30 PSG Sorinex), deployed in the classic configuration in order to obtain one ECG lead and ADInstruments hardware (Power Lab 4/30 and Bio Amplifier). During registration, the animals remained under a heating lamp to prevent the decline in body temperature. Immediately prior to registration, the animals from subgroups 2A/2B and 3A/3B received the last, fifth MLT/AMT dose, whereas the control rats were given the last injection of saline.

Once registrations were completed, the ECG signal was visually evaluated to remove ectopic beats and the remaining records were subjected to HRV analysis in both time and frequency (spectral) domain, using ADInstruments software (Chart v5.4.2) for Mac OS X Version 10.1.2. The basis for the HRV analysis is the variability of the adjacent, “normal-normal” (N-N) intervals duration, which is subject to continuous, ANS-modulated fluctuations. The other time-domain HRV parameters are secondary measures, based on mean N-N statistical parameters [18]. We analysed standard time-domain HRV parameters: the average duration of N-N intervals (mean N-N), the longest N-N interval (max N-N), the shortest N-N interval (min N-N), the global standard deviation of N-N intervals (SDN-N), the root mean square of the successive differences (rMSSD) – the successive differences being surrounding RR intervals (all in [ms]).

During the spectral HRV analysis, total power (TP) of HRV spectrum is determined along with the powers (in [ms^2]) of its basic components, i.e. the distribution of N-N intervals with respect to the cyclic ANS-modulated activities of the sinus node, associated with three main essential rhythms: in the range of very low (VLF), low (LF) and high (HF) frequencies [18]. In this analysis, we adopted the following ranges for the individual spectral components of HRV: 0.01 < VLF < 0.28 < LF <0.78 < HF < 3. These frequency ranges were similar to those used by Aubert et al. [19] (0.01 < LF < 0.74 < HF < 2.5), and Goncalves et al. [20] (0.01 < LF < 1.0 < HF < 3.0). Subjecting the the HRV spectrum to the process of normalization, we also calculated the normalized nHF and nLF values, which are regarded to reflect the selective parasympathetic and sympathetic tension, respectively. The HRV spectrum normalization is based on the calculation of the sharing of relevant com-
ponents (LF or HF) in total HRV power, excluding VLF component power. This procedure results from the difficulties of interpretation regarding the VLF component [18, 21, 22] (see below in Discussion section).

Finally, after the administration of the lethal dose of sodium pentobarbital (400 mg/kg b.w.), a cystectomy was performed to measure the bladder wet weight (BWW) and to obtain specimens for histopathological evaluation. A bladder was collected from each of the study animals, following a previous separation from the surrounding adipose tissue and voiding. According to literature, BWW may be considered an indirect measure of inflammatory remodelling of the bladder [23, 24]. Immediately after collection, bladders were weighed on an analytic scale and then placed in 4% formalin solution with PBS for further histopathological evaluation. The finally prepared microscopic sections were stained with haematoxylin and eosin (HE) to enable the histologic evaluation of the inflammation severity.

The HRV results were expressed as mean ±SD. Statistical analysis was performed using analysis of variance (ANOVA), which verifies the existence of statistically significant differences between three studied populations. Whenever statistically significant differences were demonstrated (p < 0.05 on ANOVA test), the significance of differences between paired (1-2A, 1-2B, 1-3A and 1-3B) populations was tested using the Student’s t-test. All statistical calculations were performed on values subjected to logarithmic transformation in order to ensure greater consistency of analysed HRV parameters with normal distribution.

Results
Characteristics of the studied groups
In all analysed groups, a progressive decrease in body weight, associated with the subsequent doses of the CP, was noted. On the 7th day of the experiment, the animals from subgroup 2B were characterized by the lowest body weight, while the control group by the highest one. A similar dependence was found in the case of mean body weight for all studied groups. However, these differences were not statistically significant. Detailed data are provided in Table 1.

Assessment of the collected bladders
Bladder wet weight measurement showed that the animals treated with MLT (subgroup 2A and 2B) were characterized by slightly higher BWW values compared to animals treated with AMT (subgroups 3A and 3B) and to the controls. These differences were not statistically significant. The detailed results are also given in Table 1. The histopathological examination of bladder specimens confirmed the presence of inflammatory lesions in all studied groups.

Bladders collected from control rats were characterized by a clear oedema and signs of congestion (mostly in the mucosa), along with signs of focal proliferation of fibroblasts in the mucosal lamina propria, mostly around some fine submucosal blood extravasations. Fine lymphocytic inflammatory infiltrations were visible in the vicinity of vessels of the mucosal lamina propria. Epithelium of the bladder lining demonstrated focal ulceration with signs of clear proliferation of cells. Complex papillary architecture showing anastomosis of papillae with focally irregular nuclei and few scattered mitotic figures was intensified in subgroups 3A/3B, while signs of oedema and hyperaemia were revealed in subgroups 2A/2B (MLT treatment) and in the controls; the lesions documented in subgroups 2A/2B was slightly lower than in the controls.

Results of HRV analysis
Time-domain HRV analysis
The animals treated with MLT, regardless of the MLT dose, were characterized by the highest values of mean and maximum N-N interval, with concurrently the lowest value of min N-N from all groups. The differences related to mean N-N were statistically significant. Additionally, rats from subgroups 2A and 2B were characterized by the lowest average heart rate, and the highest SDN-N and rMSSD values. These differences turned out to be statistically significant when compared to the controls. Most of the time-domain HRV parameters recorded in AMT-treated animals did not differ significantly from respective values of the controls, except for the average HR and rMSSD (these parameters were significantly lower) and mean N-N (it was higher) in animals from subgroup 3A and average

| Group 1 | 1st day – 1st dose | 3rd day – 2nd dose | 5th day – 3rd dose | 7th day – 4th dose | Mean body weight | BWB |
|---|---|---|---|---|---|---|
| Control | 288.0 ±11.8 | 284.5 ±9.1 | 283.7 ±9.4 | 281.7 ±10.6 | 284.5 ±3.6 | 181.9 ±4.6 |
| Subgroup 2A | 286.7 ±16.6 | 284.7 ±28.0 | 273.7 ±26.9 | 258.7 ±23.6 | 275.9 ±12.8 | 191.7 ±5.4 |
| Subgroup 2B | 274.7 ±6.2 | 268.0 ±10.4 | 260.0 ±8.7 | 246.0 ±9.9 | 262.2 ±12.3 | 209.6 ±6.1 |
| Subgroup 3A | 281.3 ±8.1 | 278.0 ±6.9 | 272.0 ±8.7 | 256.3 ±3.8 | 271.9 ±11.1 | 184.6 ±4.4 |
| Subgroup 3B | 279.7 ±13.3 | 279.3 ±16.7 | 276.7 ±16.3 | 267.3 ±13.6 | 275.8 ±5.7 | 192.1 ±4.3 |
HR (lower value) in rats from subgroup 3B. Detailed results of the time-domain HRV parameters, including statistical conclusions, are presented in Table 2.

**Spectral (frequency) HRV analysis**

The spectral analysis carried out in MLT-treated animals revealed statistically significant differences for the total power value of the HRV spectrum, and for all non-normalized parameters: VLF, LF and HF. They achieved significantly higher values in animals treated with MLT, at both 40 mg/kg b.w. (subgroup 2A) and 100 mg/kg b.w. (subgroup 2B), than in the controls. These differences were more pronounced in subgroup 2B, i.e. after higher MLT dose. Furthermore, the animals of this subgroup differed significantly from the controls in terms of the normalized parameters, namely higher nLF and lower nHF. A similar phenomenon was not observed in subgroup 2A, i.e. after the smaller MLT dose.

Contrary to the rats treated with MLT, animals treated with AMT did not differ significantly from the controls in terms of spectral parameters of HRV, with the exception of TP and VLF, which achieved significantly higher values in rats treated with AMT at 40 mg/kg b.w. The detailed results of HRV spectral analysis, including statistical conclusions, are presented in Table 3.

**Discussion**

Our most important finding in the analysis of HRV in animals with experimental, cyclophosphamide-induced hemorrhagic cystitis, treated with either melatonin or agomelatine, was to demonstrate that:

1. Melatonin, administered in rats with cyclophosphamide-induced cystitis, modulated ANS activity, causing an increase in global autonomic tension. The agent either proportionally stimulated both sympathetic and parasympathetic part of ANS (at a dose of 40 mg/kg b.w.) or preferentially enhanced sympathetic activity (at a dose of 100 mg/kg b.w.).

2. Agomelatine, regardless of the applied dose, did not exert significant effect on autonomic activity in animals with cyclophosphamide-induced cystitis.

3. Taking into account the fact that AMT, as melatonin receptors agonist, did not affect the functional autonomic state, it may be assumed that the modulatory effect of MLT on the ANS activity seems to result from its non-receptor pharmacodynamic mechanisms (e.g. antioxidant activity, regulating the secretion of various inflammatory mediators).

Currently, the indirect, non-invasive assessment of the autonomic nervous system activity enables the analysis of heart rate variability (HRV). The stimulation of sympathetic or parasympathetic ANS branch is reflected by changes in the HRV parameters, both time- and spectral ones. According to the commonly accepted interpretative HRV guidelines [18, 21, 22, 25], parameters such as SDN-N and rLF correlate with sympathetic activity, rMSSD, HF and rHF are associated with parasympathetic activity, and the power of LF component reflects the activity of both the sympathetic and parasympathetic part. Total power (TP) of the HRV spectrum is a marker of the global ANS activity.

The interpretation of the part of the HRV spectrum contributing to the range of very low frequency (VLF) is the most controversial. The exact mechanisms underlying the formation of that spectral component of HRV are still not completely understood. The background of VLF includes a broad array of various stimuli: thermoregulatory processes, the renin-angiotensin-aldosterone system, hemodynamics feedback delays, mechanical and central neuronal effects of breathing patterns, spinal reflexes or vascular autorhythmicity [26]. The VLF power increases during sympathetic stimulation (systemic stress, chronic heart failure, shock, vasodilatation). On the other hand, some evidence suggests that VLF may be of parasympathetic origin. Taylor et al. [27] showed that atropine almost completely abolished VLF power and other spectral components. Silva Soares et al. [28] also revealed that

| Parameter | Group 1 Control | Subgroup 2A MLT 40 mg/kg | Subgroup 3A AMT 40 mg/kg | Statistic p value 1-2A | 1-3A |
|-----------|-----------------|--------------------------|--------------------------|-----------------------|------|
| mean N-N [ms] | 160.7 ±9.9 | 171.2 ±11.3 | 170.6 ±7.2 | 0.05 | 0.02 |
| max N-N [ms] | 182.6 ±6.9 | 188.9 ±3.1 | 187.1 ±12 | NS | NS |
| min N-N [ms] | 146.2 ±8.3 | 141.7 ±4.4 | 147.1 ±9.7 | NS | NS |
| average HR [1/min] | 375.3 ±23.0 | 350.7 ±22.8 | 352.4 ±15.5 | 0.04 | 0.01 |
| SD N-N [ms] | 6.2 ±2.3 | 11.2 ±4.5 | 8.6 ±3.6 | 0.02 | NS |
| rMSSD [ms] | 4.1 ±4.2 | 9.4 ±8.6 | 1.4 ±1.8 | 0.05 | 0.05 |

| Parameter | Group 1 Control | Subgroup 2B MLT 100 mg/kg | Subgroup 3B AMT 100 mg/kg | Statistic p value 1-2B | 1-3B |
|-----------|-----------------|--------------------------|--------------------------|-----------------------|------|
| mean N-N [ms] | 160.7 ±9.9 | 175.8 ±9.6 | 167.7 ±5.2 | 0.01 | NS |
| max N-N [ms] | 182.6 ±6.9 | 188.8 ±5.5 | 184.9 ±7.0 | NS | NS |
| min N-N [ms] | 146.2 ±8.3 | 147.9 ±12.8 | 149.7 ±11.2 | NS | NS |
| average HR [1/min] | 375.3 ±23.0 | 342.2 ±18.9 | 358.0 ±11.0 | 0.01 | 0.03 |
| SD N-N [ms] | 6.2 ±2.3 | 10.1 ±5.2 | 5.5 ±2.5 | 0.04 | NS |
| rMSSD [ms] | 4.1 ±4.2 | 10.9 ±8.5 | 5.2 ±4.3 | 0.03 | NS |
VLF band may be driven by parasympathetic modulation. They demonstrated that stimulation with pyridostigmine (reversible cholinesterase inhibitor) produced a strong increase in VLF power. Therefore, VLF may also depend on the presence of parasympathetic outflow.

Having taken into account the abovementioned interpretative HRV guidelines, the results of our experiment support the hypothesis that the administration of melatonin in CP-HC rats produces modulatory effect in relation to the ANS activity, the more pronounced the higher MLT dose was applied. Administration of MLT (100 mg/kg b.w., subgroup 2B) was reflected by evident sympathetic predominance. This was manifested by an increase in SDN-N and nLF and, indirectly, by an increase in VLF and LF (assuming sympathetic background of these two parameters).

Contrary to MLT therapy, administration of agomelatine did not result in any significant changes in both the global autonomic activity and tones of its particular parts. As mentioned above, agomelatine is a melatonin receptor agonist. Therefore, it may be assumed that modulatory properties of MLT did not result from the MT1/2 receptor stimulation, but rather from non-receptor, additional pharmacodynamic mechanisms of MLT, related to the antioxidant and inflammation-regulatory properties of this agent. The stimulation of the sympathetic part after MLT treatment can also result in amelioration of the adrenergic-mediated (via receptors) bladder overactivity (CP-HC is associated with some LUTS originating from bladder over-contractility).

The abovementioned findings, pointing to a role of MLT in the regulation of the autonomic function in CP-HC, are also consistent with the results of our previous studies which evaluated influence of MLT and AMT on bladder motility in an experimental CP-HC model [14]. We revealed that MLT (75 or 100 mg/kg b.w.) ameliorated bladder overactivity observed in the course of experimental CP-HC. On the contrary, AMT applied at the same dose, even aggrava-

### Table 3. Spectral-domain HRV analysis results (mean ±SD; NS – non-significant). Statistical analysis performed for logarithmic values

| Parameter | Group 1 Control | Subgroup 2A MLT 40 mg/kg | Subgroup 3A AMT 40 mg/kg | Statistic p value 1-2A | 1-3A |
|-----------|-----------------|--------------------------|--------------------------|------------------------|------|
| TP [ms²]  | 11.1 ±14.1      | 49.5 ±35.5               | 19.6 ±6.9                | 0.05                   | 0.01 |
| VLF [ms²]| 7.4 ±8.7        | 39.1 ±45.2               | 17.2 ±6.9                | 0.05                   | 0.01 |
| LF [ms²] | 1.4 ±2.1        | 5.5 ±5.8                 | 1.2 ±1.1                 | 0.05                   | NS  |
| HF [ms²] | 2.4 ±3.5        | 5.1 ±4.3                 | 1.3 ±1.2                 | 0.04                   | NS  |
| nLF [n.u.]| 40.0 ±20.6      | 40.9 ±18.9               | 37.7 ±26.0               | NS                     | NS  |
| nHF [n.u.]| 60.0 ±20.5      | 59.1 ±18.9               | 62.3 ±26.0               | NS                     | NS  |

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| Parameter | Group 1 Control | Subgroup 2B MLT 100 mg/kg | Subgroup 3B AMT 100 mg/kg | Statistic p value 1-2B | 1-3B |
|-----------|-----------------|---------------------------|---------------------------|------------------------|------|
| TP [ms²]  | 11.1 ±14.1      | 58.6 ±47.1                | 17.8 ±20.3                | 0.01                   | NS  |
| VLF [ms²]| 7.4 ±8.7        | 47.3 ±38.2               | 11.0 ±15.3                | 0.01                   | NS  |
| LF [ms²] | 1.4 ±2.1        | 6.2 ±5.4                 | 1.8 ±2.7                 | 0.02                   | NS  |
| HF [ms²] | 2.4 ±3.5        | 5.1 ±4.3                 | 4.9 ±8.0                 | 0.03                   | NS  |
| nLF [n.u.]| 40.0 ±20.6      | 55.9 ±5.9                | 32.2 ±20.4               | 0.04                   | NS  |
| nHF [n.u.]| 60.0 ±20.5      | 44.1 ±5.9                | 67.8 ±20.4               | 0.03                   | NS  |
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and showed that the beneficial effects of this compound may be partially caused by the reorganization of the ANS status with the sympathovagal balance shifted towards sympathetic overdrive. The increased sympathetic function and global autonomic tension, observed in experimental CP-HC after a large MLT dose, improve bladder compliance and can contribute to alleviating of some LUTS resulting from bladder overactivity. Our findings, as well as the results of the abovementioned previous studies, justify further research on the beneficial role of MLT in the CP-HC. The future studies may lead to official approval of MLT as yet another new uroprotector reducing the toxicity of cyclophosphamide.

The authors declare no conflict of interest.

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