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Spectral heart rate variability in rats with cyclophosphamide-induced hemorrhagic cystitis treated with cyclooxygenase inhibitors

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Abstract: The pathogenesis of cyclophosphamide-induced hemorrhagic cystitis (CP-HC) is complex, involving the impact of many systemically and locally released agents on autonomic nervous system (ANS) activity, that affects bladder functioning.
The purpose of our study was to provide an indirect evaluation of ANS functional status in experimental CP-HC model, involving prostaglandin synthesis block resulting from administration of cyclooxygenase inhibitors.
The ANS activity was estimated through the spectral analysis of heart rate variability (HRV) in CP-HC rats divided into three study groups: 1-control, 2-treated with meloxicam (MLX) that preferentially blocks COX-2, and 3-treated with piroxicam (PRX) that inhibits COX1 and 2 activity.
In animals treated either with MLX or PRX, the percent distribution of the spectrum in relation to components of very low (VLF) and low (LF) frequency was not different from the control group. PRX-treated group displayed nearly two times lower percent share of the high frequency (HF) component compared to the control. Moreover, an increase of the normalized LF (nLF) value with simultaneous reduction of the normalized HF (nHF) value were noted in PRX-treated rat with no change of these parameters for MLX-treated rats.
The HRV analysis in CP-HC rats receiving PRX, indicated a functional reorganization manifested by reduced parasympathetic activity and increased sympathetic tonus. A partial prostaglandin synthesis block caused by COX-2 inhibitor (meloxicam) caused no significant changes of evaluated HRV parameters compared to the control. Assessing functional changes of the ANS caused by prostaglandin synthesis block it should be stated that prostaglandins synthesized by the constitutive COX-1 isoform seem to maintain the parasympathetic activity, which may be associated with the cholinergic anti-inflammatory pathway and resolution of inflammation in course of cyclophosphamide-induced cystitis.

Key words: cystitis, autonomic nervous system, heart rate variability, piroxicam, meloxicam.

Introduction

Urinary bladder overactivity symptoms are observed both in functional and organic morbidities of the bladder. One of clinical circumstances associated with development of symptoms of secondary overactive bladder syndrome (OAB) is hemorrhagic cystitis caused by therapy with drugs belonging to the group of oxazaphosphorins, especially with cyclophosphamide (CP). The drug is still commonly used in therapy of lymphoproliferative conditions, Hodgkin disease and neoplastic diseases (e.g. small-cellular lung cancer or breast cancer), and in therapy of other diseases, including nephrotic syndrome or visceral lupus [1]. However, the use of cyclophosphamide is associated with risk of numerous and multi-systemic adverse effects, including the above mentioned hemorrhagic cystitis with bladder overactivity [2]. Pathogenesis of cyclophosphamide-induced hemorrhagic cystitis (CP-HC) is a complex one, associated with systemic biotransformation of oxazaphosphorins and endovesical release of a toxic metabolite — acrolein — secondary initiating release of numerous preformed and de novo synthesized pro-inflammatory mediators, and finally inducing cellular damage. A detailed description of CP-HC pathogenesis is presented in one of our reviews [3].

CP-HC prevention and therapy is based on parallel administration of cytoprotective agents, mainly mesna (a donor of sulfhydryl groups that react with the double bond in acrolein, transforming that oxazaphosphorine metabolite into a less toxic product) [4]. Other, currently studied cytoprotective agents potentially effective in CP-HC therapy involve phytopharmacological compounds (belonging to groups of flavonoids, vitanoloids, steroidal saponins, vegetable alkaloids and organic sulphur compounds) [3] and prostaglandins analogues [5]. It is generally known that prostaglandins are synthesized locally in the stomach and exert their cytoprotective action towards the gastric mucosal barrier, as well as mucous barrier and mucosal perfusion [6]. Prostaglandins are also released by vesical urothelium and demonstrate similar cytoprotective properties [7]. At the same time, there are reports from studies on pathogenesis of idiopathic OAB, suggesting that prostaglandins increase the contractility activity of the detrusor. Therefore, those compounds may be perceived as one of paracrine mediators, that may contribute to bladder overactivity [8].

Considering the above mentioned premises associated with a potential, alleviating effect of prostaglandins administered in CP-HC, it is worth explanation if those compound are also able to modulate the activity of the autonomic nervous system (ANS), and, if those potential changes in autonomic regulation could contribute to their protective properties in course of CP-HC. Hence, our purpose was to indirectly assess the autonomic activity in experimental CP-HC by spectral heart rate variability (HRV) method, utilizing effects resulting from prostaglandin synthesis inhibition by non-steroid anti-inflammatory drugs (NSAIDs). Moreover, using a non-selective inhibitor of COX-1 and COX-2, or a preferential COX-2 inhibitor, we were trying to determine if the potential effect on ANS activity was demonstrated by constitutive or inflammatory-induced prostaglandins.

Materials and methods

Ethical aspects of the experiment

The medical experiment described in this paper was approved by the 1st Local Bioethical Committee for Animal Experiments in Krakow (approval's No 124/2013).

Study animals

The experiment was conducted on thirty 8-weeks old female Wistar rats obtained from the central animal house of the Pharmacy Faculty at the UJCM in Krakow. At arrival to the animal house of the Chair of Pathophysiology UJCM rats were placed in 5 cages, with unlimited access to water and standard laboratory feed (Labofeed, Kcynia). The experiment was conducted following a 10-day acclimatization period. After that time animals were randomly assigned to study groups.

Study groups and general outline of the experiment

The study was carried out in animals with experimentally induced CP-HC, divided into three groups of 10 participants each: 1 — control (CP-HC animals without additional treatment), 2 — CP-HC animals receiving the preferential cyclooxygenase 2 inhibitor — meloxicam and 3 — animals receiving the non-specific cyclooxygenase inhibitor — piroxicam. Baseline and end-of-experiment body weight was determined in each study group. Water and feed consumption during the experiment were not monitored, and animals had an unlimited access to both. All study animals had cyclophosphamide-induced cystitis induced for 7 days, and during this period groups 2 and 3 received additionally cyclooxygenase inhibitors: MLX in the group 2, and PRX in the group 3, while control animals were sham treated (0.9% saline solution). On the day 8 of the experiment all rats had heart rate variability (HRV) recordings, and then, following administration of a lethal dose of pentobarbital — cystectomy was performed in order to determine bladder wet weight (BWW) and for further histopathological evaluation. Characteristics of study animals and BWW results are presented in the Table 1, below.

Table 1. Characteristics of study groups.

	Rats' body weight		Statistic	Bladder wet weight [mg]	Statistic	Bladder wet weight/body weight ratio [%]	Statistic
	Starting [g]	Final [g]					
CP-HC Group 1	210.3 ± 12,0	178.7 ± 16.1	–	170 ± 60	–	0.095 ± 0.04	–
CP-HC + MLX Group 2		187.3 ± 10.7	NS	150 ± 20	NS	0.080 ± 0.01	NS
CP-HC + PRX Group 3		156.3 ± 19.6	NS	130 ± 40	p = 0.05	0.083 ± 0.02	NS

NS — non significant

Experimental model of hemorrhagic cystitis

All rats were *intraperitoneally* administered cyclophosphamide (CP) at the dose of 75 mg/kg b.w. (at the first, third, fifth and seventh day of the experiment) in order to induce a chronic cyclophosphamide-induced cystitis. According to numerous literature reports, CP administered four times in dosing scheme described above, results in development of CP-HC [9, 10]. We have also confirmed the fact in our previous studies using that method and performing pathomorphological evaluation of bladders, and verifying with urodynamic records of bladder overactivity [11]. Cyclophosphamide was obtained in the crystalline form from Sigma Aldrich (cyclophospamide monohydrate) and was prepared in form of an aqueous solution *ex temporae*, before administration of subsequent doses.

Therapy with cyclooxygenase inhibitors

MLX and PRX were administered respectively to animals in groups 2 and 3, in an hour following the administration of CP. Commercially available formulas were used (meloxicam — MOVALIS, Boehringer Ingelheim, ampoules 15 mg/1.5 ml; piroxicam — FELDENE, Pfizer, ampoules 20mg/ml). Rats in the control group received i.p. injections with normal saline in the mean volume corresponding to PRX/MLX doses. Following an appropriate dilution with water for injection, a defined dose of study agents was intraperitoneally administered, similarly to CP. We have used the dose of 10 mg/kg b.w. of piroxicam, considering experience gained by other researchers who used PRX at the dose of 10 mg/kg b.w. [12] or 5 mg/rat with body weight of approx 400 g [13]. Selection of meloxicam dose of 5 mg/kg b.w. was based on reports published by Laird *et al*. study [14] who used the compound in rats at doses ranging between 0.1 and 4 mg/kg b.w., everyday for 5 days, and reports published by Bourque *et al*. [15], who administered MLX at the dose of 1–2 mg/kg b.w. every day for 3 days, subcutaneously. On that basis we concluded that our meloxicam administration regimen involving i.p. injection at the dose of 5 mg/kg b.w., every other day, will be finally a similar dosing scheme.

Animals lost during the study

In the control group (group 1) two animals died before administration of the last, fourth CP dose. Therefore, finally the group consisted of 8 animals. Other rats demonstrated normal activity, but their condition deteriorated progressively under the unfavorable, systemic effect of CP. The deterioration was manifested by body weight loss measured in relation to the baseline (Table 1).

All animals with CP-HC treated with MLX (group 2) survived until the end of the experiment, and in the group 3 (CP-HC+PRX) three animals died. Therefore, 7 animals were studied finally in that group. In both groups 2 and 3, similarly to the control group, body weight reduction was observed, being a result of unfavorable effect of CP. A relatively poorer condition of rats in the group 3 (manifested by the number of dead animals) could be also associated with potential adverse effects of PRX to alimentary tract mucosa (Table 1).

ANS function estimation by HRV recording

On the day 8 of the experiment, an indirect assessment of the autonomic nervous system activity using the heart rate variability, based on electrocardiographic records, was performed in all study groups. ECG was registered in rats under general urethane anesthesia (1200 mg/kg b.w.; i.p.) at rest, for 20 minutes. Before the ECG registration rats' abdomens were carefully shaved in order to achieve epidermal abrasion, and a standard ECG gel was applied. The record was performed using pediatric Ag/AgCl electrodes (EG-S30 PSG Sorimex) placed according to the classic configuration, to achieve one ECG lead. During the registration rats were placed under a heating lamp to protect them from reduced body temperature.

After the registration, the obtained ECG signal was evaluated visually in order to remove any extra-sinus stimulations and the record was analyzed for heart rate variability. The starting point in the HRV analysis is the assessment of duration of neighboring „normal-normal" (N-N) intervals, that undergo constant, autonomically-controlled fluctuations [16]. Currently, HRV is considered to be the best, non-invasive, indirect method for the autonomic functional status estimation, allowing to carry out two types of analysis: time (adopted especially for long-term ECG recordings) and spectral ones [16, 17]. We decided to base our reasoning on spectral (frequency) analysis that results from subjecting of the N-N intervals variability to fast-Fourier transformation or autoregression methods. In this procedure, finally powers of components of the, so called, HRV spectrum are estimated, that is distribution of RR intervals variability changes in relation to cyclic, ANS-modulated stimulating activity of the sinus node associated with three principal rhythms: very low frequency (VLF), low frequency (LF), and high frequency (HF) [16, 17]. For spectral HRV analysis we adopted the following ranges for individual HRV spectral components: 0.18<VLF<0.28<LF<0.78<HF<3. The frequency ranges adopted by us were similar to those used by Aubert *et al*. [18] (0.19<LF<0.74<HF<2.5) and Goncalves *et al*. [19] (0.10<LF<1.0<HF<3.0) Moreover, we assessed the percent share of power of individual spectral components (VLF [%], LF [%], HF [%]) in relation to the total power of the spectrum, and LF/HF ratio, and values of standardized nLF and nHF parameters.

Collection of bladders and their histopathological assessment

Following HRV recording animals were sacrificed with a lethal dose of pentobarbital (Morbital, Puławy, Poland; 100mg/kg b.w.) for weight and histopathological analysis of their bladders. The bladder was collected following a previous separation from the surrounding fatty tissue and voiding. According to the literature data, measurement of the bladder wet weight (BWW) may be treated as an indirect evidence of the bladder reconstruction induced by inflammation [20, 21]. Directly after collection, bladders were weighed on an analytic scale and then placed in 4% formalin solution with PBS for further histopathological evaluation. The detailed description of the histopathological procedure was placed in one of our previous paper [22].

Statistical analysis of results

The statistical analysis was performed using the statistical suite R.3.0.2. (R Foundation for Statistical Computing, Vienna, Austria).

First, existence of statistically significant differences between three study groups was verified with the variance test analysis (ANOVA). Then, if statistical significance was demonstrated for an individual analyzed parameter ($p < 0.05$ in ANOVA), differences between groups 1 and 2 (control CP-HC vs. CP-HC+MLX), and 1 and 3 (control CP-HC vs. CP-HC+PRX) were analyzed with the t-Student test. Both tests (ANOVA and t-Student) were done on logarithms of obtained HRV results. The procedure was aimed at assurance of higher conformity of those parameters with the normal distribution.

Results

Bladder Wet Weight (BWW) and histopathological evaluation or urinary bladders

The BWW measurement indicated that the parameter reached its highest value in control animals, and the lowest in piroxicam-treated ones and this difference was of a statistical significance. However, referring the measured BWW valued to the final body weight of animals and expressing that association as a percent index, similar results were obtained in all study groups, and no statistically significant differences were found (Table 1).

The histopathological analysis revealed non-specific vesicular inflammatory changes in all study groups, with signs of congestion, edema, together with presence of lymphocytes in mucosal lamina propria (features of *papillosis urothelialis focalis*).

HRV spectral analysis

The spectral analysis of HRV records of animals treated with MLX (group 2) demonstrated that percent distribution of basic, non-standardized spectral components in those animals was nearly identical as in the control group, without any statistically significant differences. Similarly, values of normalized nLF and nHF parameters found for the group 2 were not statistically significantly different from those in the control group.

In the group 3 (CP-HC+PRX), similarly to the group 2, the percent distribution of the spectrum in relation to components VLF and LF was not statistically significantly different from the control group, but — contrary to the group 2 — the percent share of the HF component was nearly two times lower compared to the control, and the difference was statistically significant. Moreover, statistically significant increase of the nLF value with simultaneous reduction of the nHF value were noted in the group 3 rats (treated with PRX) compared to the control.

Results of basic spectral parameters and their percent distribution in relation to the total HRV spectrum power, along with the statistical analysis are presented in the Table 2, below, and Figures 1 and 2 show changes of normalized nLF and nHF parameters.

Table 2. Results of the HRV spectral analysis.

Parameter	CP-HC Group 1	CP-HC+MLX Group 2	CP-HC+PRX Group 3	Statistic	
				Groups 1–2	Groups 1–3
VLF [%]	70.09 ± 12.63	69.02 ± 27.26	77.16 ± 7.99	NS	NS
LF [%]	11.41 ± 4.19	10.83 ± 7.54	12.86 ± 4.52	NS	NS
HF [%]	18.51 ± 9.87	20.15 ± 25.62	9.98 ± 4.16	NS	p = 0.05
LF/HF	0.82 ± 0.55	0.97 ± 0.65	1.14 ± 0.39	NS	NS

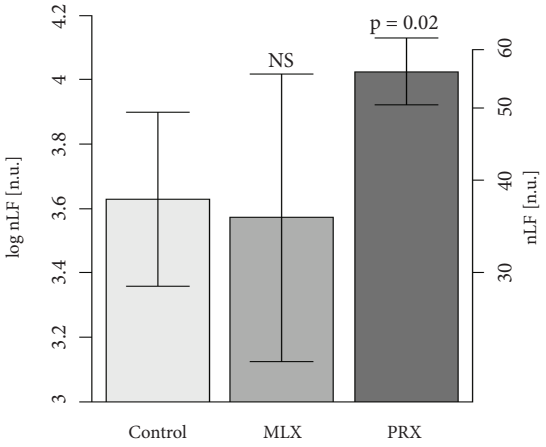
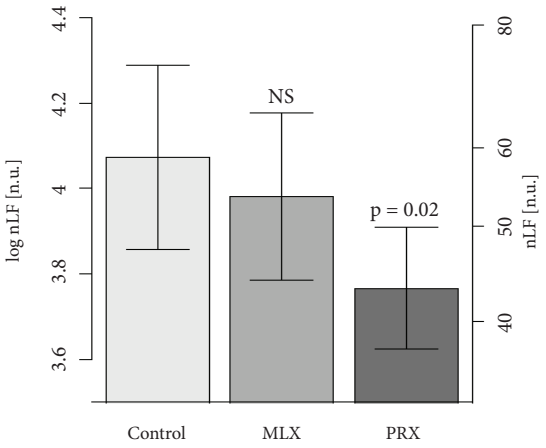


Fig. 1. Changes of the normalized nLF parameter value in study groups.



For both figures: A box height stands for the mean value of a logarithm of a parameter in the particular group, and whiskers present the 95% confidence interval for the logarithm.
Both scales: raw and logarithm, were marked to facilitate the interpretation.
A result of the statistical analysis was marked in relation to the control group (p value or labelled as NS — non significant).

Fig. 2. Changes of the normalized nHF parameter value in study groups.

Discussion

Considering the unquestioned endocrine activity of the urothelium and its role in bladder function control, it seems that prostaglandins are a necessary element of homeostasis of the organ [8, 23]. They are synthesized locally in the urinary bladder, both in basic (synthesis with constitutive COX-1) and pathological (with COX-2 induced by, among other, inflammation) conditions. COX-1 expression is found in the urothelium, whereas both COX-1 and COX-2 are expressed in the muscular layer [24]. There are some species-specific differences in the prevailing type of prostaglandins released locally in the bladder. However, it may be generally stated that the following prostaglandins are present in the bladder: PGE2, PGF2α, PGD2 and PGI2 [8].

It is generally accepted that those compounds are principal pro-inflammatory mediators, co-responsible for development of all classic symptoms of an acute inflammation (local reddening, warming, edema, pain as well as systemic reaction — fever). However, on the other hand, there is also an opinion that COX-2 pathway-dependent prostaglandins are also engaged in mechanisms associated with resolution of an inflammation. It is possible that those compounds demonstrate their anti-inflammatory effect exerting a beneficial influence on urothelium and muscular coat through several mechanisms. Generally speaking, in presence of prostaglandins cellular membranes become strengthened and tightened. That results in disappearance of edematous changes. Part of prostaglandins stimulate platelet aggregation and local vasoconstriction, leading to reduced hematuria. Moreover, PGE2 demonstrates cytoprotective properties (similar to those in the stomach), thus protecting from intensification of urothelium damage [7, 8].

Those reports undermine the basic dichotomy of the above mentioned division into constitutive, homeostatic prostaglandins synthesized with COX-1, and COX-2-induced, pro-inflammatory prostaglandins. Those observations also question the paradigm of using selective COX-2 inhibitors as a modern anti-inflammatory therapy, for many years perceived as safer compared to classic non-selective NSAIDs [25].

Pleiotropic properties of prostaglandins involve also their paracrine modulation of the autonomic system. Therefore, our aim was to determine the functional status of the autonomic nervous system in the experimental CP-HC model, with blocked synthesis of prostaglandins. Spectral HRV analysis performed in the group of rats with CP-HC treated with MLX demonstrated no statistically significant differences, contrary to the group treated with PRX. In that group we have demonstrated a functional rearrangement of the ANS, with marked percent reduction of the HF component. Those changes were accompanied by reduced nHF and increased nLF values. According to the commonly accepted interpretation criteria, both the non-standardized HF component, and the standardized nHF parameter reflect tension of the parasympathetic part of the ANS, while nLF is an exponent of the sympathetic activity [16, 17]. Therefore, our results indicate that administration of piroxicam (but not meloxicam) is associated with reduced parasympathetic activity. That conclusion may be also supported by the tendency of changes observed within the VLF component of the HRV spectrum. Underlying mechanisms of that component remain unclear, and that is why its interpretative significance is not as unequivocal, as it is in case of the HF component.

However, majority of researchers tend to accept the hypothesis that the VLF component is also a marker of sympathetic stimulation associated with activation of the RAA system, thermoregulation and activity of chemoreceptors [16, 26]. Whereas the percent increase of the VLF component observed by us in the group 3 was not statistically significant compared to the control group, combined with simultaneous nLF value increase the trend may reflect a parallel intensification of the sympathetic activity following the administration of PRX.

According to the classification presented in 2008 [27], piroxicam used by us is an compound belonging to the agents blocking both COX isoforms, and meloxicam belongs to the preferential COX-s inhibitors. According to other broad reviews, meloxicam is classified even as a selective COX-2 inhibitor [28], with consensus among researchers in relation to non-selectivity of piroxicam towards COX1 and 2 block [29]. Hence, taking into consideration our results, it should be concluded that prostaglandins synthesized by COX-1 and not by COX-2, demonstrate the ability to modulate the autonomic activity.

The effect of revealed changes in the autonomic activity on the inflammatory condition remains a separate question. If inhibition of prostaglandins synthesized by COX-1 results in relative increased sympathetic and reduced parasympathetic activity, it should be expected that, conversely, prostaglandins themselves intensify the parasympathetic and reduce the sympathetic activity. The arguments discussed above is partly consistent with results of our another experiment. Recently, we have examined the impact of PGE and PGF2α prostaglandin analogues on ANS activity, also estimated with HRV method, in the same experimental CP-HC model [22]. We revealed that animals treated with those compounds were characterized by an increase of HRV total power spectrum. Moreover, CP-HC rats treated with PGF2α analogue demonstrated an increase of HF power. According to the HRV interpretative guidelines it may suggest a parasympathetic activity predominance in this group of animals (on the other hand, however, we did not manage to get a similar conclusion when considering the results of standardized nLF and nHF components of HRV spectrum) [22]. Such changes in autonomic regulation mat reflect cholinergic mechanisms associated with inhibition of activity of inflammatory cells and release of various cytokines — the process is referred to as cholinergic anti-inflammatory pathway. It was demonstrated that acetylcholine released from parasympathetic fibers acts on nicotine receptors of immunocompetent cells (particularly macrophages), leading to reduction of their activity and resolution of inflammation [30]. Thus, maintenance of the parasympathetic activity by constitutive prostaglandins may contribute to alleviation of inflammation through the mechanism of the cholinergic anti-inflammatory pathway. That would be consistent with reports regarding a potential application of prostaglandins as compounds mitigating urotoxicity of cyclophosphamide [5]. Moreover, it should be also stated that demonstration of the favoring effect of prostaglandins on the parasympathetic part would constitute also another evidence of the role of those compounds in pathogenesis of idiopathic bladder overactivity (idiopathic OAB syndrome), because detrusor contractility increases with cholinergic stimulation of muscarinic receptors [7, 8]. Moreover, that would also justify the use of cyclooxygenase inhibitors (NSAIDs) in pharmacotherapy of idiopathic OAB syndrome.

We are aware of limitations of our study: autonomic activity assessment was based solely on indirect HRV method, without support from biochemical analysis. Besides, our

reasoning regarding the modulatory effect of COX1/COX 2 prostaglandins on ANS is indirect and based on HRV records obtained in conditions of inhibition of their synthesis. Also, we administrated cyclooxygenase inhibitors (as well as prostaglandin analogues, studied in our previously published paper [22]) systemically (i.p.), not intravesically. However, we wanted to maintain the same route of administration as in the case of CP and to assess the modulatory effects of several PRX/MLX doses of the ANS function in "chronic" conditions (our goal was not to study single NSAIDs dose administered intravesically in restrained animals).

Moreover, in the histopathological evaluation of bladders, no significant alleviation of inflammation or differences in BWW were observed despite the therapy with PRX/MLX. That seemingly contradicts the final conclusions. In our opinion, the fact may be a result of too short duration of the therapy and resignation from everyday dosing of PRX/MLX, sufficient for induction of the modulating effect on the ANS, but insufficient for the expected, anti-inflammatory tissue rearrangement.

Summing up, results of our HRV analysis in the experimental model of cyclophosphamide-induced hemorrhagic cystitis indicate a functional rearrangement of the ANS occurring following the block of prostaglandin synthesis with a non-selective cyclooxygenase inhibitor — piroxicam. The rearrangement was expressed by reduced parasympathetic activity and increased sympathetic tension. A partial block of prostaglandin production caused by a COX-2 inhibitor — meloxicam brought no significant changes in the ANS function. Therefore, assessing the ANS functional changes caused by prostaglandin synthesis block, it should be stated that COX-1-mediated prostaglandins maintain the parasympathetic activity in experimental cyclophosphamide-induced hemorrhagic cystitis. A cholinergic stimulation may be expressed by activation of the cholinergic anti-inflammatory pathway, and in that mechanism constitutive prostaglandins may be co-responsible for resolution of inflammation in cyclophosphamide-induced cystitis.

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Conflict of interest

None declared.

The authors contributed to the following parts of the submitted work

ŁD — design and performing research, analyzing data, writing the paper
AB — assistance by the study agents administration, the care of the animals during the experiment
KC — analyzing data
PT — critical review and final correction of the manuscript

The list of abbreviations used in the text (in alphabetical order)

ANS — Autonomic Nervous System
BWW — Bladder Wet Weight
COX — Cyclooxygenase
CP — Cyclophosphamide
CP-HC — Cyclophosphamide induced haemorrhagic cystitis
HF — High Frequency component in HRV spectrum
HRV — Heart Rate Variability
LF — Low Frequency component in HRV spectrum
MLX — Meloxicam
nHF — normalized High Frequency component
nLF — normalized Low Frequency component
N-N — Normal-Normal interval (in ECG recording)
NSAIDs — Non Steroidal Antiinflammatory Drugs
OAB — Overactive Bladder
PRX — Piroxicam
VLF — Very Low Frequency component in HRV spectrum

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