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THE INFLUENCE OF OXAZAPHOSPHORINES ALKYLATING AGENTS ON AUTONOMIC NERVOUS SYSTEM ACTIVITY IN RAT EXPERIMENTAL CYSTITIS MODEL

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Abstract: The oxazaphosphorines alkylating agents (cyclophosphamide; CP and ifosfamide; IF) are often used in common clinical practice. However, treatment with CP/IF is burdened with the risk of many adverse drug reactions, especially including hemorrhagic cystitis (HC) that is associated with bladder overactivity symptoms (OAB). The HC pathophysiology is still not fully displayed; it seems that autonomic nervous system (ANS) functional abnormalities play important role in this disturbance. The aim of our study was to reveal the potential ANS differences in rat experimental HC model, evoked by CP and IF by an indirect ANS assessment - heart rate variability (HRV) study. We carried out our experimental research in three essential groups: control group (group 1), cyclophosphamide-induced HC (CP-HC; group 2) one and ifosfamide-induced HC (IF-HC; group 3) one. CP was *i.p.* administrated four times in dose of 75 mg/kg body weight while IF-treated rats received *i.p.* five drug doses; 50 mg/kg body weight. Control rats were administrated *i.p.* vehicle in appropriate volumes as CP/IF treated animals. HRV studies were performed the next day after the last oxazaphosphorines dose. Standard time- and spectral (frequency) domain parameters were estimated. We confirmed the HC development after both CP/IF in macroscopic assessment and bladder wet weight measurement; however, it was more aggravated in CP-HC group. Moreover, we demonstrated HRV disturbances, suggesting ANS impairment after both studied oxazaphosphorines, however, consistent with the findings mentioned above, the autonomic dysfunction was more emphasized after CP. CP treatment was also associated with changes of non-normalized HRV spectral components percentage distribution - a marked very low frequency - VLF [%] increase together with low frequency -LF [%] and high frequency - HF [%] decrease were observed. Taking into consideration the next findings, demonstrating the lack of both normalized power spectral components (nLF and nHF) values, the VLF percentage change seems to be of special meaning. IF produced smaller autonomic disturbances, and gentler bladders histological abnormalities comparing to CP. However, similar to CP, VLF [%] relative augmentation together with LF [%] and HF [%] drop accompanied the global ANS activity decrease. Additionally, in the case of IF treatment, a slight trend of nLF increase with nHF decrease was noted, suggesting the possible functional rearrangement between sympathetic (nLF) and parasympathetic (nHF) tension. It seems possible that the vagal withdrawal and - as a consequence - sympathetic overactivity, reflected by VLF [%] enlargement and HF and LF [%] diminishing (as well as LF and HF values decrease), may be an evidence of impaired anti-inflammatory cholinergic pathway, aggravating bladder inflammatory lesions. To sum up, our study showed ANS impairment in both CP- and IF-evoked experimental HC that was reflected in HRV recordings. HRV study, thus, may be considered to be a diagnostic tool for CP/IF treated patients, estimating autonomic abnormalities, associated with the HC development risk and its clinical course.

Keywords: oxazaphosphorines, cyclophosphamide, ifosfamide, hemorrhagic cystitis, autonomic nervous system, heart rate variability (HRV)

Abbreviations: ANS - autonomic nervous system, CP – cyclophosphamide, CP-HC – cyclophosphamide-induced HC, HC – hemorrhagic cystitis, HF – high frequency (a HRV spectrum component), HRV – heart rate variability, IF – ifosfamide, IF-HC – ifosfamide-induced HC, LF – low frequency (a HRV spectrum component), nHF – normalized HF, nLF – normalized LF, OAB – overactive bladder, RMSSD – the square root of the mean squared difference of successive normal-normal (R-R) intervals, SDNN – the standard deviation of all normal-normal (R-R) intervals, VLF – very low frequency (a HRV spectrum component).

Oxazaphosphorines belong to a group of alkylating agents with a broad spectrum of antitumor activity in man. Cyclophosphamide (CP) is a key substance, that possesses high activity against both many experimental tumors and in clinical conditions; however, oxazaphosphorines group contains

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also other agents: ifosfamide, trofosfamide, sufosfamide and mafosfamide (1, 2).

Cyclophosphamide is one of the most frequently used oxazaphosphorine agent and although effective alone in susceptible malignancies, is more frequently used concurrently or sequentially with other antineoplastic drugs. The following malignancies are often susceptible to CP treatment: Hodgkin's disease, lymphocytic lymphoma (nodular or diffuse), mixed-cell type lymphoma, histiocytic lymphoma, Burkitt's lymphoma, multiple myeloma, chronic lymphocytic leukemia, chronic granulocytic leukemia (but it is usually ineffective in acute blastic crisis), acute myelogenous and monocytic leukemia, acute lymphoblastic (stem-cell) leukemia in children (when given during remission is effective in prolonging its duration). Moreover, CP is used in management of breast cancer, mycosis fungoides, neuroblastoma, ovarian adenocarcinoma, and retinoblastoma. CP is also used in non-malignant diseases, mostly in biopsy proven "minimal change" nephrotic syndrome in children, but should not be used as primary therapy (3).

Ifosfamide (IF) is the other predominantly used oxazaphosphorine agent. It is a synthetic analogue of cyclophosphamide. IF is mostly used in combination with certain other approved antineoplastic agents, is indicated for third line chemotherapy of germ cell testicular cancer. Additionally, since ifosfamide's first approval, a large variety of malignancies have been studied to asses their responses. Clinical experience shows that it is also useful for other medical problems. Although these uses are not included in product labelling in most countries, ifosfamide is used in certain patients with bone cancer (including Ewing's sarcoma), in female ovarian, breast, cervix or endometrium cancers as well as in head and neck, lungs cancers in both sexes. Moreover, IF is considered to be effective in lymphomas, neuroblastoma, thymoma and other cancer of the thymus and Wilms' tumor (a cancer of the kidneys occurring mainly in children) (4).

It has been revealed, that cytotoxic action of all oxazaphosphorines is closely related to the reactivity of the 2-chloroethyl substituent group, which in turn is linked with the basicity of the central nitrogen atom. It was discovered that after oxazaphosphorines administration, these chemotherapeutics are subject to complex biotransformation processes before they can exert their alkylating cytotoxic properties. To sum up, the metabolic pathway consists of three major steps; activation, toxification and deactivation. The initial activation results in hydroxylation of the C-4 atom, which is effectuated by the mixed system of liver oxidases. At a further stage of toxification, the formed 4-hydroxyoxazaphosphorines get metabolized with a spontaneous elution of acrolein and a formation of diamine derivatives of phosphoric acid with alkylating properties. Simultaneously, because of the fact that toxification reaction is a rate-limiting enzymatic step, a reversible or an irreversible deactivation may take place. Due to the saturation of enzymes synthesizing alkylating metabolites, reactions of dehydrogenation (oxidation) to aldo-oxazaphosphorine forms may occur alternatively, which formations are then further converted into 4-keto or carboxy derivatives that are excreted in the end. This deactivation pathway hyperactivity may explain the presence of oxazaphosphorine-resistant tumors (1, 5, 6). According to Ross et al. (7), who formulated their concept over sixty years ago, the cytotoxic action of alkylating agents is based on the alkylation of nucleophilic centres of biomolecules (such as the N7 position of guanine in DNA). Thus, the intracellular release of alkylating agents leads to the DNA polymerases inhibition and specific DNA alkylation (by the covalent bonding) with subsequent DNA polymerization depletion ("suicidal inactivation") (1, 5, 6, 8).

Oxazaphosphorine alkylating compounds are still widely used cytostatic agents, however, they are not devoid adverse reactions – from mild or moderate disturbances to severe, even life-threating. They have already been reported by Coggins et al. in 1960 (9).

Both CP and IF adverse drug reactions are multiple, including disturbances originating almost from all systems, such as hematologic (myelosuppression with possible life-threatening opportunistic infections or sepsis), gastrointestinal (moderate to severe emesis, anorexia, and, less frequently, abdominal discomfort, pain or diarrhea), dermatologic (alopecia in at least 50% of patients treated with cyclophosphamide and 83% with ifosfamide; typically reversible), respiratory (interstitial pulmonary fibrosis, pneumonitis, dyspnea), endocrine (amenorrhea associated with decreased estrogen and increased gonadotropin in women, oligo- or azoospermia associated with normal testosterone and increased gonadotropin in men) and neurological abnormalities (ototoxicity and peripheral neuropathy, asthenia, dizziness, depression, or headache) (10, 11).

Among various, possible oxazaphosphorines-induced side effects mentioned above, genitourinary side effects including hemorrhagic or nonhemorrhagic cystitis, associated with bladder overactivity

accompanied by irritative voiding, urinary frequency, dysuria, urgency, incontinence, nocturia seem to be one of the most important. Moreover, for ifosfamide, renal tubular acidosis, Fanconi syndrome, renal rickets and acute renal failure and hematuria occurring in 6 to 92% of patients has been reported. Ifosfamide-induced renal tubular necrosis has also been reported as most threating side effect (12, 13). The prevalence of HC development as a result of CP/IF treatment was reported to be as high as about 70%. Life-threatening HC with uncontrolled hemorrhage is estimated at about 4% (14).

The pathophysiological mechanism of HC development is complex, based on oxazaphosphorines-derived metabolites inflammatory response activation.

In summary, according to Korkmaz, Topal and Oter (14), the most likely pathomechanism of oxazaphosphorines (more exactly: acrolein-induced) hemorrhagic cystitis, can be described using several details. In the first step, acrolein enters into the uroepithelium and initiates its injury by itself, that is deepened by acrolein-evoked both ROS and NO overproduction. Moreover, acrolein is responsible for further uroepithelial damage intensification by some intracellular factors such as NF-κB and AP-1 inducement. These compounds cause the overexpression of genes encoding proinflammatory cytokines (TNF-α and IL-1) and a further increase in the synthesis of ROS and NO. At a later stage, cytokines leave the uroepithelium and spread to detrusor smooth muscle and bloodstream. It seems that the key point in HC development plays peroxynitrite synthesis that is formed from ROS and NO. Peroxynitrite attracts cellular macromolecules (lipids, proteins, DNA), further exaggerates bladder damage. As a result, damage to the integrity of cells and tissues occurs, manifesting itself in the form of histological and morphological abnormalities, such as swelling, bleeding and ulcerations. Thus, our current knowledge about pathogenesis of oxazaphosphorines-induced hemorrhagic cystitis is evolved. There is no doubt that this is a complex inflammatory process with several cytokines, free radicals and non-radical molecules and that all these components of a pathophysiological description must be taken into consideration when looking for more effective chemopreventive compounds against CP / IF-induced cystitis (14). The more detailed pathophysiological description of HC development as well as oxazaphosphorines mechanism of action we gave in one of our reviews (15).

Thus, the cytotoxic action oxazaphosphorine therapy may be complicated by bladder functional abnormalities induction resulting in bladder overactivity; so called - iatrogenic overactive bladder (OAB) syndrome.

On a margin, the cyclophosphamide administration to rats is regarded to be one of the oldest, but still used experimental OAB model, described for the first time by Cox et al. (16).

OAB is a clinical entity, characterized due to International Continence Society guidelines (2002 and 2006) as urgency with or without urgency incontinence, usually with increased daytime urination frequency and nocturia (17, 18). The OAB pathogenesis is complex, involving several neurogenic and myogenic disturbances, with paying the special attention to abnormal afferent and efferent bladder innervation, abnormal paracrine urothelium activity and disturbed bladder smooth cells myoelectrical activity. Thorough the description of OAB pathophysiology is exceeding the framework of this work, an interested reader will find more details in other articles (19-21), including those ones of our authorship (22).

It is worth stressing, that the idiopathic OAB is developing at the lack of any organic disturbances being the possible causative OAB agents, while cystitis accompanying oxazaphosphorines therapy results in similar clinical manifestation. Thus, rat oxazaphosphorines model is regarded to reflect clinical idiopathic OAB.

Aim of the study

The aim of our study was to reveal the potential differences of autonomic nervous system activity in hemorrhagic cystitis undergoing with bladder overactivity, evoked by two oxazaphosphorines: cyclophosphamide (CP) and ifosfamide (IF). Nowadays, the best non-invasive method of ANS indirect assessment is the heart rate variability (HRV) study. HRV is the temporary variation of R-R intervals observed in ECG recording that is modulated by autonomic influences (23).

EXPERIMENTAL

The study protocol was approved by the First Local Ethic Committee in Kraków (agreement decision 126/2010).

Animals and studied groups

The animals were obtained from the central laboratory. Rats were allowed an acclimatization period of two weeks in groups of five per cage upon their arrival at the animal house of the Pathophysiology Department. The animals were housed at room tem-

perature, with 12/12 hours day-night cycle, with standard food (Labofeed, Kcynia) and *ad libitum* water. During the third week, being a principal experiment period, all animals were fed with high-energetic fodder to minimalize the unfavorable effects of administrated cytotoxic agents on alimentation.

After the acclimatization period (14 days), the animals were randomized into studied groups. We carried out our experimental research in three essential groups: control group (group 1), cyclophosphamide-induced HC (CP-HC; group 2) one and ifosfamide-induced HC (IF-HC; group 3) one. Each group consisted of ten 10-week-old female Wistar rats; thus the experiment employed 30 animals.

CP-HC and IF-HC model

Both cyclophosphamide and ifosfamide were injected intraperitoneally (*i.p.*) to produce a model of chemically induced hemorrhagic cystitis (HC) with bladder overactivity. In general, depending on CP/IF dose and route of administration, the so-called "acute overactive bladder" model (after high single dose), or a chronic case (after several doses) may be evoked (24, 25). We conducted our study on CP-HC model caused by cyclophosphamide (Sigma-Aldrich; 75 mg/kg body weight), administered *i.p.* four times every third day during one week (starting with the 1st day and then on the 3rd, 5th and 7th day of a week), while IF-HC model was induced by five time, daily administration of 50 mg/kg body weight of ifosfamide. Adjusting IF-HC protocol to the CP-HC group, IF were administrated in 3rd, 4th, 5th, 6th and 7th day. According to literature, this resulted in a chronic chemical bladder inflammation (24, 25). In IF-HC group, all rats finished the study, while two animals did not survive CP therapy, thus, in the end, we studied 8 CP-HC rats. We also observed progressive hematuria, which appeared in all remaining CP-HC subjects after the last CP dose. This phenomenon occurred only in two of animals receiving IF. On the 8th day, HRV recording was registered in both CP and IF treated rats and after these procedures the animals were euthanized by pentobarbital (Morbital, Biowet, Puławy) overdose (100 mg/kg body weight).

Control group

The studied animals received saline *i.p.* injection from 1st till 7th day of the experiment (except the 2nd day, because no rat received drug this day) in similar volumes as those administrated in CP and IF rats. HRV recordings were also performed on the 8th day of the experiment.

HRV studies

ECG recordings were performed during 20-min rest periods under urethane anesthesia (1200 mg/kg body weight; Sigma-Aldrich) in each studied animal. This anesthetic agent was chosen based on literature reports suggesting a proportional (up to the applied dose) impact on tonic activity of both the sympathetic and parasympathetic ANS, and a relatively small influence on cardiac reflexes (26, 27). After terminating ECG registration and extrasinusal extopics elimination, HRV analysis was performed. Standard spectral (frequency; TP, VLF, LF, HF – all in [ms×ms] and normalized nLF and nHF in [n.u.]) parameters were calculated. The frequency range for respective spectral components was set at: $0.18 < VLF < 0.28 < LF < 0.78 < HF < 3$; and commonly accepted interpretation criteria were admitted. Results were presented as the mean values ± SD.

Urinary bladder assessment

Bladders were collected in both CP-HC and IF-HC rats, as well as in the controls, after ECG recordings and administration of lethal morbital dose (100 mg/kg body weight). We carefully collected bladders with small proximal urethra to make a gross visual assessment of the inflammatory changes. Moreover, after gentle urine evacuation and bladders dry on the gauze, the samples were weighted to

Table 1. Characteristics of studied rats.

Assessed parameters	Control group 1	CP-HC group 2	IF-HC group 3	Statistic – p value		
				Group 1-2	Group 1-3	Group 2-3
Starting body weight	192.86 ± 4.85	189.70 ± 10.13	187.43 ± 6.8	NS	NS	NS
Final body weight	295.50 ± 8.21	157.0 ± 16.05	169.14 ± 8.17	0.001	0.001	NS
Bladder wet weight	0.137 ± 0.064	0.168 ± 0.06	0.137 ± 0.04	NS	NS	NS
Bladder wet weight / final body weight ratio	0.045 ± 0.023	0.108 ± 0.04	0.081 ± 0.02	0.01	0.01	NS

NS = Not significant

settle bladder wet weight. According to the literature, bladder wet weight is regarded to be an indirect parameter, reflecting the cystitis and edema intensification (28-30). We also calculated bladder wet weight to final body weight ratio to express the bladder weight participation independently on the rat individual differences. The statistical analysis of obtained results was performed, comparising control to both studied groups as well as CP-treated (group 2) to IF-treated (group 3) animals, using parametric Fischer-Snedecor test with $\alpha = 0.05$. The final results together with statistical estimation are given in Table 1.

In both studied groups (CP-HC and IF-HC), urinary bladders were red and swollen. These histological lesions were more stressed in CP treated animals. Moreover, bladders obtained from CP-HC group, in most cases were covered by abundant serosal petechial suffusions. Histological inflammatory changes were less pronounced in IF treated rats. Contrary to CP-HC group, we did not reveal intrabladder hematomas in IF treated animals. The control rats had normal in visual inspection bladders.

Statistical analysis

The statistical assessment of the results obtained in paired studied groups (CP-HC *vs*. control and IF-HC *vs*. control) was performed after expressing them as ln values, again using parametric Fischer-Snedecor test with $\alpha = 0.05$. The expression of the values of spectral parameters in the form of natural logarithms was the consequence of the lack of their normal distribution. The null (H0) hypothesis of equality of analyzed parameter variations in the two studied populations was verified *versus* the alternative hypothesis (H1) of their inequality (the presence of statistically significant differences).

RESULTS

Animals' weight

The starting weight was similar in all studied animals. At the end of the experiment (three weeks; 14 days of acclimatization and one week of oxazaphosphorines or vehicle administration), the final body weight differed in individual groups. An increase in final body weight was observed in control rats (about 53%; from 192.9 to 295.5 g), while in two remaining groups we noticed a progressive body weight fall, more pronounced in CP-treated rats (mean body weight loss was about 17% - from 189.7 to 157.0 g in this group, whereas IF-treated animals displayed smaller body weight decrease – about 8%; from 184.43 to 169.14 g). The cause of body weight loss in oxazaphosphorines treated rats was the deterioration of their general condition together with observed diminished food intake comparing to control.

Bladder wet weight in studied groups

The bladder wet weight did not differ significantly in all studied groups, although this parameter achieved the highest value in CP-HC rats. The ratio: bladder wet weight / final body weight was the lowest in control animals, elevated in IF-treated participants and the highest in CP-treated ones. These differences were of statistical meaning when separately compared control to CP-HC and IF-HC groups. This finding reflects more intensified inflammatory changes and edemas in bladders tissue in animals receiving CP / IF. Comparing CP-HC and IF-HC rats, these differences were not so clear anymore, however, in our opinion, it is worth underlying that the general tendency suggesting the highest tissue lesions in CP-treated rats was revealed.

The detailed description of the results mentioned above is given in Table 1.

HRV time-domain parameters

Most of calculated time-domain HRV parameters were similar in all studied groups, without essential differences in control and both treated animals. Control rats were characterized by higher range, mean NN and rMSSD comparing to those ones treated with CP. The only difference relating to control and IF-treated groups was also rMSSD, which – contrary to the findings mentioned above – was higher in IF-treated rats.

HRV spectral-domain parameters

The spectral (frequency) HRV analysis showed many essential differences with statistical significance among studied groups.

In general, the values of all non-normalized HRV spectral parameters were the highest in control group. The clearly marked trend of the all non-normalized spectral parameters fall was demonstrated in both oxazaphosphorines treated groups. These parameters achieved the lowest values in CP-treated group.

Moreover, we calculated the percentage of individual power spectral components in total HRV power in all analyzed groups. In control animals, the HRV spectrum was characterized by the highest VLF percentage, amounting almost a half of total spectrum additionally with almost two-times higher HF percentage comparing to LF one. In both CP and IF treated animals, the disproportion between three

non-normalized spectral components comparing to their distribution in control rats was showed. We found that the percentage of VLF markedly increased, achieving almost three fourth of total spectrum. Moreover, LF percentage decreased about the half in both CP/IF treated animals, comparing to control ones. The HF [%] also differed considerably in both oxazaphosphorines treated participants, amounting lower values.

Contrary to the findings mentioned above, considering the results of normalized spectral parameters we did not reveal significant differences between control and treated rats. The nLF and nHF values were almost the same in control and CP-treated rats. In IF-HC group, the only essential difference was related to nLF, which was higher than in control one (simultaneously nHF was slightly diminished comparing to the control, although it was not statistically significant).

The detailed results mentioned above are given in Table 2.

DISCUSSION

The main findings revealed in our study were:

1. Both cyclophosphamide and ifosfamide impaired autonomic nervous system functioning. We revealed that these oxazaphosphorines agents diminished global autonomic activity that was more profound after CP and this drug also produced more intensified bladders histological lesions comparing to IF.

2. Moreover, CP treatment was associated with changes of non-normalized HRV spectral components percentage distribution - a marked VLF [%] increase together with LF [%] and HF [%] decrease were observed. Taking into consideration the next findings, demonstrating the lack of both normalized nLF and nHF values, the VLF percentage change seems to be of special meaning. In our opinion, autonomic impairment after CP was manifested by decrease of total autonomic tension with relative VLF functional predominance, due to systemic

Table 2. Time- and spectral domain HRV analysis after 4-times cyclophosphamide (CP; 75 mg/kg body weight) and 5-times ifosfamide (IF; 50 mg/kg body weight) administration in rats.

HRV parameters	Control Group 1	CP-HC Group 2	IF-HC Group 3	STATISTICAL p value (for ln HRV parameters values)	
Time-domain HRV parameters				**Group 1-2**	**Group 1-3**
max NN [ms]	188.8 ± 0.28	185.4 ± 7.0	188.6 ± 0.8	NS	NS
min NN [ms]	139.3 ± 0.37	145.1 ± 9.5	141.8 ± 5.6	NS	NS
range [ms]	49.5 ± 0.5	40.2 ± 16.1	46.8 ± 5.4	**0.001**	NS
mean NN [ms]	163.4 ± 10.3	157.9 ± 12.4	164.4 ± 9.4	**0.05**	NS
average HR [1/min]	368.3 ± 22.6	381.9 ± 29.1	365.9 ± 20.9	NS	NS
SDNN	8.7 ± 2.7	6.6 ± 3.2	12.7 ± 5.0	NS	NS
rMSSD	13.0 ± 7.3	5.5 ± 5.2	19.3 ± 15.2	**0.05**	**0.02**
Spectral-domain HRV parameters					
TP [ms²]	36.1 ± 17.8	10.6 ± 15.6	28.7 ± 36.9	**0.04**	**0.01**
VLF [ms²]	19.2 ± 15.7	7.0 ± 9.2	15.9 ± 20.8	**0.02**	NS
LF [ms²]	5.4 ± 2.5	1.4 ± 2.5	3.8 ± 7.2	**0.05**	**0.04**
HF [ms²]	11.5 ± 8.9	2.2 ± 4.0	9.1 ± 13.7	**0.001**	**0.04**
LF/HF	0.7 ± 0.6	0.6 ± 0.6	1.1 ± 0.7	NS	**0.003**
nLF [n.u.]	36.6 ± 16.0	33.6 ± 21.6	45.2 ± 21.1	NS	**0.001**
nHF [n.u.]	63.4 ± 16.0	66.4 ± 21.6	54.8 ± 21.1	NS	NS
VLF [%]	47.1 ± 26.6	76.2 ± 13.8	71.6 ± 28.2	**0.009**	**0.009**
LF [%]	16.4 ± 5.5	8.6 ± 6.3	8.3 ± 5.4	**0.001**	**0.001**
HF [%]	36.5 ± 21.5	15.2 ± 9.1	20.2 ± 27.6	**0.05**	**0.01**

NS = Not significant

(including cardiovascular) stress followed by cytotoxic CP action.

3. IF produced smaller autonomic disturbances, and gentler bladders histological abnormalities comparing to CP. However, similar to CP, VLF [%] relative augmentation together with LF [%] and HF [%] drop accompanied the global ANS activity decrease. Additionally, in the case of IF treatment, a slight trend of nLF increase with nHF decrease was noted, suggesting the possible functional rearrangement between sympathetic (nLF) and parasympathetic (nHF) tension.

During normal sinus rhythm, the heart rate varies from beat to beat. Heart rate variability results from the dynamic interplay between multiple mechanisms that regulate the instantaneous heart rate (31). Among them, autonomic nervous system is the most prominent factor determining cardiac functions and its rate, thus the heart rate variability study, reflecting ANS impact on heart rate is nowadays considered to be a good tool for ANS activity assessment. As it was mentioned in the introduction, the basic data for the HRV calculation is the sequence of time intervals between normal heart beats. The heart is innervated by both parasympathetic fibers, that cause the interbeat interval becomes longer and the sympathetic ones, causing the time between heart beats to become shorter. The fluctuations between normal beats, expressed by main HRV parameter – mean NN [ms] and some derived statistical parameters are the time-domain HRV analysis (31, 32). Moreover, additional insight into the nature of heart rate fluctuations may be gained by analyzing the fluctuations in the frequency domain analysis, based on the fast Fourier transformation method. The frequency – spectral HRV analysis provides information of how power (variability) distributes as a function of frequency as well as yields clinical interpretation of obtained results. Many physiological studies showed that the parasympathetic influences are pervasive at the frequency range 0.15–0.4 Hz (> 6 s cycle length) of the heart rate power spectrum, whereas the sympathetic influences switch at about 0.04–0.15Hz (2.5 to 6.0 s cycle length). The most important premises enabling to set HF component as a marker of parasympathetic activity were the findings that respiratory related heart rate variation occurs at a high frequency (typically around 0.25 Hz) and can be abolished by vagal blockade. On the other hand, cyclical variation occurring in association with changes in baroreceptors activity is noted at a frequency of about 0.10 Hz that was also correlated with direct measures of sympathetic nerves activity. These premises suggested sympathetic-mediated LF origin. However, vagal blockade or excitation also produced some modification of LF component power. Therefore, a common agreement exists that high frequency (HF) band represents parasympathetic influences while low one (LF) has a mixed – sympathetic and parasympathetic character. There is also a possibility to express pure sympathetic or parasympathetic activity by normalized nLF or nHF; respectively, as the reference of LF or HF powers to total HRV power with the another spectral component – VLF omission (23, 33, 34). The short-term HRV spectrum also contains very low frequencies – VLF (0.0033–0.04 Hz; > 25 s cycle length), however, it is not possible to elucidate whether more heightened sympathetic or parasympathetic activity contributes to VLF power, due to still unknown physiological background of this HRV component development. VLF is regarded as reflecting circadian and neuroendocrine rhythms, thermoregulatory processes, renin-angiotensin system activity, and hemodynamic feedback delays. Although, the meaning of VLF is still not clearly understood, most HRV authorities recommend considering this component as a marker of sympathetic activity, also reflecting general systemic stress (23). However, on the other hand, there are also different opinions, regarding VLF as the marker of parasympathetic stimulation. According to Taylor et al. (35), VLF depends primarily on the presence of parasympathetic outflow. These authors showed that atropine almost completely abolished VLF power and other spectral components, suggesting that also HRV present in the VLF band is driven by parasympathetic modulation. Silva Soares et al. (36) demonstrated in experimental study that stimulation with pyridostigmine (reversible cholinesterase inhibitor) produced a strong increase in VLF. Therefore, the prognostic value of VLF oscillations may also derive from the fundamental importance of parasympathetic mechanisms.

Bringing back our results to introduced interpretative HRV assumptions above, we can ascertain that oxazaphosphorines-induced cystitis is characterized by global decrease of autonomic tension, affecting both sympathetic and parasympathetic branch. We revealed TP decrease, as well as LF, HF and VLF drop, suggesting both sympathetic and parasympathetic withdrawal. On the other hand, however, the normalized nLF and nHF values were the same in control and CP/IF treated rats (except nLF in IF-HC animals that was higher). Thus, in our opinion, in the light of these settlements stated above, the relative percentage changes of main spec-

tral components pay the special attention, especially those relating to VLF [%]. We revealed in both CP-HC and IF-HC groups the considerable proportional part increase of VLF in HRV spectrum. Taking under the attention the VLF interpretation criteria, this finding may be treated, however, as a reflection of enlarged sympathetic activity due to inflammatory changes and blood proinflammatory cytokines occurrence. It also seems to be interesting to confront our HRV results with the cholinergic-antiinflammatory pathway theory. This physiological phenomenon is associated with the nervous modulation of pro-inflammatory cytokines release from the immune-competitive cells. An excessive expression of such kind of proteins contributes to the larger tissue inflammatory damage. The anti-inflammatory, compensatory mechanism exists that is based on the vagal nerve activation. It has already been demonstrated that stimulation of vagal nerve significantly inhibits one of the most powerful proinflammatory cytokine – tumor necrosis factor (TNF-α) release (37). The mechanism of this vagal inhibition involves acetylocholine – a neurotransmitter of the vagal fibers and the nicotinic acetylocholine receptor expressed on cytokine-producing macrophages. The administration of acetylocholine to the α7 subunit of the nicotinic receptor with subsequent intracellular signal transduction through the affecting of nuclear factor – κB, results in a reduced TNF-α production, together with other pro-inflammatory agents: interleukin 1 and 6 (38, 39).

There is a relationship between heart rate variability and inflammatory markers. The majority of studies reported that parasympathetic tone is inversely related to inflammatory markers (38). Low HF (reflecting pure parasympathetic tone) and LF – a spectral component reflecting both sympathetic and parasympathetic tension, are often associated measure linked to inflammatory intensification. It can be explained based on the cholinergic antiinflammatory arc theory mentioned above – decreased vagus nerve activity and associated loss of the tonic inhibitory influence of cholinergic antiinflammatory action on immune cells response, may significantly enhance proinflammatory cytokine release (39). We revealed similar trend in our study – in both CP/IF treated rats, both HF and LF were lower than in control, thus suggesting possible disturbed cholinergic antiinflammatory pathway. Taking again into consideration our next findings demonstrating no differences between normalized nLF and nHF values and, on the other hand, the relative increase of VLF percentage part in total HRV spectrum, it seems, that this parameter may reflect diminished parasympathetic activity and relative sympathetic predominance.

CONCLUSION

According to us, it seems possible that the vagal withdrawal and – as a consequence – sympathetic overactivity, reflected by VLF [%] enlargement and HF and LF [%] diminishing (as well as LF and HF values decrease), may be an evidence of impaired anti-inflammatory cholinergic pathway, aggravating bladder inflammatory lesions. These findings support the clinical evidence indicating that when vagus nerve activity is deficient, inflammation is excessive (39). This is also consistent with other reports, demonstrating altered HRV in the presence of systemic infection, correlated with its severity (40). It also seems rational that the activity of parasympathetic branch, being a part of whole autonomic tension estimation, should be performed in each patient receiving oxazaphosphorines cytotoxic therapy. Thus, similar to hemoglobin HbA1C level assessment in diabetes (as a factor reflecting glycemic control), the heart rate variability study could be developed to monitor the functional autonomic state during oxazaphosphorines treatment to detect patients subpopulation with the low vagal activity, being particularly threatened with the development of clinically more intensified HC.

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