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Salivary exoglycosidases in the detection of early onset of salivary gland involvement in rheumatoid arthritis*

Ślinowe egzoglikozydazy w wykrywaniu wczesnego uszkodzenia gruczołów ślinowych w przebiegu reumatoidalnego zapalenia stawów

Authors' Contribution:

- A** Study Design
- B** Data Collection
- C** Statistical Analysis
- D** Data Interpretation
- E** Manuscript Preparation
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Summary

Introduction:

The aim of the present study was to evaluate the possibility of making use of the specific activity of N-acetyl- β -hexosaminidase, its isoenzymes and β -glucuronidase – potential indicators of salivary gland damage – in the detection of early onset of salivary gland impairment in RA, which is also demonstrated by xerostomia.

Material/Methods:

For this purpose RA xerostomic salivary patients (unstimulated salivary flow >0.1 mL/min) were compared with RA xerostomic hyposalivary patients (unstimulated salivary flow ≤ 0.1 mL/min), RA patients without xerostomia (unstimulated salivary flow >0.1 mL/min) and generally healthy controls (unstimulated salivary flow >0.1 mL/min, without xerostomia). Salivary N-acetyl- β -hexosaminidase, its isoenzymes A and B, and β -glucuronidase specific activity were determined according to the Marciniak et al. method. The protein content in the unstimulated saliva was determined by the bicinchoninic acid method.

Results:

In xerostomic rheumatoid arthritis patients, the specific activity of salivary β -glucuronidase and isoenzyme A was significantly higher than in the healthy controls but the specific activity of salivary N-acetyl- β -hexosaminidase, its isoenzyme B and β -glucuronidase was significantly lower than in xerostomic hyposalivary rheumatoid arthritis patients.

Conclusions:

We suggest a simple, safe and cheap method for the determination of exoglycosidases as a useful tool for the diagnosis of early stages of salivary gland involvement in rheumatoid arthritis.

Key words:

lysosomal exoglycosidases • saliva • rheumatoid arthritis • xerostomia

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INTRODUCTION

Rheumatoid arthritis (RA) is a chronic immunological systemic disease of connective tissue affecting about 1% population of the world [18]. Main symptoms of RA are connected with destructive inflammation of the joints [21, 22, 23], but RA frequently presents extra-articular symptoms of peri- and myocarditis, lung fibrosis, vasculitis, reactive amyloidosis with renal lesions, and impairment of eyes and salivary glands [19, 23]. The salivary gland involvement in RA has been known for a long time in the form of secondary Sjögren's syndrome (sSS) [13, 18, 19]. sSS is an autoimmune disorder characterized by exocrine gland destruction and dysfunction, concerning mainly the lachrymal and salivary glands, leading to reduced tear and saliva (unstimulated ≤ 0.1 mL/min) secretion [13, 18, 19]. sSS is usually accompanied by xerostomia [13, 18, 19]. Xerostomia is a subjective feeling of oral dryness, a symptom that may be accompanied by an objective marker of salivary gland dysfunction – hyposalivation (unstimulated salivary flow ≤ 0.1 mL/min) [5, 6, 11]. However, it was shown that in the initial phase of sSS and salivary gland impairment in the course of RA, sialometry might not show any significant changes in the salivary flow rates (unstimulated salivary flow > 0.1 mL/min), whereas saliva composition may have been significantly changed by autoimmune inflammation [17, 30]. It was demonstrated that xerostomia may be present at the early stages of the salivary gland involvement in RA when the disease is incipient [30].

Another marker of salivary gland dysfunction may be increase in the catabolism of glycoconjugates. Glycoconjugates include glycoproteins and glycolipids [29], constituting salivary gland cell membranes, as well as polysaccharide chains of glycosaminoglycans and proteoglycans constituting the glycoprotein extracellular matrix [31]. In the catabolism of salivary glycoconjugates are involved lysosomal N-acetyl- β -hexosaminidase (HEX) and its isoenzymes A and B (HEX A, HEX B), and β -glucuronidase (GLU) [31]. Glycosaminoglycans are broken down by endoglycosidases and its shorter fragments are successively cleaved at the non-reducing end by GLU and HEX, the most active of lysosomal exoglycosidases [7]. The activity of salivary exoglycosidases in healthy people

is low. It was reported that the elevation in the specific activities of salivary exoglycosidases reflects their higher production/release connected with breakdown of damaged fragments of salivary gland tissues [28]. Bierć et al. [3] noted a significant increase in the activities of β -glucuronidase, β -galactosidase, and HEX with its isoenzymes in salivary gland tumors. Waszkiewicz et al. [27] claimed that the determination of the activity of HEX and its isoenzymes may be a useful marker of the salivary gland dysfunction caused by a large single dose of ethanol. Borzym-Kluczyk et al. [4] showed that the salivary HEX and its isoenzymes may be valuable indicators of pleomorphic adenoma of the salivary gland.

The aim of the present study was to assess the possibility of making use of the specific activity of HEX, its isoenzymes and GLU – potential indicators of salivary gland damage – in the detection of early onset of salivary gland impairment in RA, which is thought to be demonstrated by xerostomia. For this purpose RA xerostomic salivary patients (unstimulated salivary flow > 0.1 mL/min) were compared with RA xerostomic hyposalivary patients (unstimulated salivary flow ≤ 0.1 mL/min), matched RA patients without xerostomia (unstimulated salivary flow > 0.1 mL/min) and healthy controls.

MATERIALS AND METHODS

The study was conducted on RA patients in the Medical University Hospital, Białystok, Poland. All RA patients fulfilled the American College of Rheumatology revised criteria for RA [2]. All participants fulfilled a questionnaire with questions on symptoms of eye and oral dryness, as used in the American-European classification criteria for Sjögren's syndrome [25]. Each participant had determined the unstimulated salivary flow rate and Schirmer I-test. RA patients and healthy women were excluded from the study if they had objective (Schirmer I-test < 5 mm/5 min) or subjective (positive response to at least one question associated with eye dryness) eye dryness, used medications which could cause oral dryness, smoked cigarettes, had diabetes mellitus, hypertension, HCV or HIV infection. Sixteen xerostomic salivary RA women (RA NSX) selected for the present study had at least one positive response to the question associated with oral dryness

[25] and unstimulated salivary flow >0.1 mL/min. Two age- and sex-matched RA groups were also selected: 16 RA non-xerostomic women (unstimulated salivary flow >0.1 mL/min) with no positive response to the questions associated with oral dryness (RA NS), and 16 RA xerostomic hyposalivary women (unstimulated salivary flow ≤ 0.1 mL/min) with at least one positive response to the questions associated with oral dryness (RA HSX). Sixteen healthy control women, matched in age, with no objective or subjective oral (unstimulated salivary flow >0.1 mL/min, without xerostomia) or eye dryness (Schirmer I-test >5 mm/5 min, without xerophthalmia) were recruited in the Department of Conservative Dentistry, Medical University of Białystok, Poland.

Written informed consent was obtained from each patient and healthy participant after the aims and methodology of the study were explained. The study was approved by the Ethics Committee of the Medical University of Białystok (permission number R-I-002/282/2007).

Clinical examinations were performed by one dentist (A.Z.) under standardized conditions in the Pedodontics Department at the Medical University of Białystok, in a dental chair using portable equipment with fiberoptic light, suction device and compressed air. All examinations were done using diagnostic dental tools (dental mirror, probe and periodontal probe).

Following WHO criteria, the dental status of each woman was determined using the DMFT index. Gingival status was examined using the gingival index GI, and oral hygiene was determined using the oral hygiene index - simplified (OHI-S) (Table 1). In 25 patients, the inter-rater agreements between the examiner (A.Z.) and another dentist (J.Sz.) were assessed. The reliability for: DMFT was r=0.99; GI was r=0.97; OHI-S was r=0.94.

Each RA patient underwent a clinical examination, which included blood tests, laboratory markers of inflammation covering the erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), and rheumatoid factor (RF) evaluated by the Waaler-Rose test (WR), ANA, anti-SSA and anti-SSB (Table 1).

Unstimulated whole saliva (UWS) collection

The participants were instructed to refrain from food and beverages, except water, for two hours before the saliva collection. All salivary samples were collected between 8 a.m. and 10 a.m., so that circadian influences would be minimized. During saliva collection, the patient was seated on a chair and protected from gustatory and other stimulation. The whole saliva was collected into a plastic tube placed on ice by the spitting method, under standardized conditions [20]. The secretion rate of unstimulated whole saliva was measured for 15 min. Immediately after collection,

Table 1. Clinical data of the RA and the control groups (mean±SD)

	RA NS (n= 16)	RA NSX (n= 16)	RA HSX (n= 16)	Control (n= 16)
Age (yr)	50.5±12.43	53.28±9.9	51.45±11.3	55.5±8.4
OHI-S	0.42±0.21	0.45±0.4	0.44±0.4	0.48±0.53
GI	0.59±0.6	0.54±0.55	0.87±0.75	0.68±0.42
DMFT	21.46±3.9	22.35±5.1	26.2±2.33	18.86±5.0
ESR (mm)	59.56±11.02	56.4±16.5	64.9±25.45	nd
CRP(mg/mL)	37.21±24.3	28.59±32.19	52.94±37.47	nd
RF (%)	72	86	100	nd
WR	245.38±190.74	257.56±237.56	345±197.95	nd
Disease duration (yr)	11.59±7.15	13.11±2.29	11.25±8.95	nd
Schirmer-I test				
right eye (mm/5min)	16±9	18±6	15±5	18±4
left eye (mm/5min)	17±8	19±7	18±9	20±7
anti-SSA	not detected	not detected	not detected	nd
anti-SSB	not detected	not detected	not detected	nd
ANA (%)	not detected	not detected	25	nd

(n- number of subjects; SD- standard deviation; GI- gingival index; OHI-S- oral hygiene index- simplified; DMFT- Decay, Missing, Filled teeth; ESR- erythrocyte sedimentation rate; CRP- C- reactive protein; RF- Rheumatoid Factor; WR- Waaler- Rose test; nd- not determined; RA NS- non-xerostomic rheumatoid arthritis patients; RA NSX- xerostomic rheumatoid arthritis patients, RA HSX- xerostomic hyposalivary rheumatoid arthritis patients).

salivary samples were centrifuged at 3 000 x g for 20 minutes at 4°C to remove cells and debris. The resulting supernatants were divided into 50 µl portions, frozen and kept at -80°C until analyzed.

Analytical methods

Activities of total HEX and its isoenzymes HEX A, HEX B as well as GLU in the supernatants of saliva were determined in duplicate by the Marciniak et al. method [15] with p-nitrophenyl-β-D-N-acetylglucosaminopyranoside (Sigma, St Louis, MO, USA) as a substrate for the determination of total HEX, HEX A and HEX B and p- nitrophenyl-β-D-glucuronide (Fluka) as a substrate for GLU. Heat-stable HEX B was measured after selective heat denaturation of thermolabile HEX A. HEX A was calculated from the difference between the total HEX and HEX B activity. Measurement of p-nitrophenol released by the HEX and GLU activities was carried out at 405 nm using the microplate reader Elx800™.

The protein content in saliva was determined in duplicate by the bicinchoninic acid (BCA) method with bovine albumin as a standard (PIERCE BCA Protein Assay Kit). All analyses were performed in duplicate.

Statistical analysis was performed using Statistica 10.0 (StatSoft, Cracow, Poland). ANOVA Kruskal-Wallis, median and LSD (for stomatological and laboratory parameters) tests were used to study the significant differences between groups. Spearman correlation coefficients were used to determine the association between two variables. Statistical significance was defined as $p < 0.05$.

RESULTS

The HEX specific activity in the saliva of RA xerostomic patients (RA NSX) showed a very strong tendency to increase in comparison to the healthy controls ($p=0.09$) but did not differ significantly, as compared to non-xerostomic RA patients (RA NS) ($p=0.82$). The specific activity of HEX in saliva of RA hyposalivary xerostomic patients (RA HSX) was significantly higher in comparison to RA non-xerostomic patients (RA NS, $p<0.001$) and RA xerostomic (RA NSX, $p<0.001$) patients and the healthy controls ($p<0.001$). The specific activity of HEX in the saliva of non-xerostomic patients (RA NS) and the healthy controls was similar (Fig. 1A).

The HEX A specific activity in the saliva of RA xerostomic patients (RA NSX) was significantly higher as compared to the healthy controls ($p=0.04$) and did not differ significantly as compared to the RA non-xerostomic (RA NS) patients ($p=0.82$). The HEX A specific activity in the saliva of RA hyposalivary xerostomic patients (RA HSX) was significantly higher as compared to the RA non-xerostomic patients (RA NS) ($p<0.001$) and healthy controls ($p<0.001$) and it was higher, although insignificantly, in comparison to RA xerostomic patients (RA

NSX, $p=0.41$). The HEX A specific activity in the saliva of RA non-xerostomic patients (RA NS) and the control group was similar ($p=0.84$) (Fig. 1B).

The specific activity of HEX B in the saliva of RA hyposalivary xerostomic patients (RA HSX) was significantly higher in comparison to the RA non-xerostomic (RA NS, $p<0.001$), RA xerostomic (RA NSX, $p<0.001$) and healthy control patients ($p<0.001$). The specific activity of HEX B in the saliva of RA NS and RA NSX patients and the healthy controls did not differ significantly (Fig. 1C).

The GLU specific activity in the saliva of RA xerostomic patients (RA NSX) was significantly higher in comparison to the healthy controls ($p=0.03$) and it was almost at the same level as in the saliva of non-xerostomic RA patients (RA NS) ($p=0.98$). The GLU specific activity in the saliva of RA hyposalivary xerostomic patients (RA HSX) was significantly higher in comparison to the RA non-xerostomic patients (RA NS), RA xerostomic patients (RA NSX) and healthy controls ($p<0.001$). The GLU specific activity in the saliva of the non-xerostomic RA patients (RA NS) and healthy controls was similar ($p=0.58$) (Fig. 1D).

The salivary flow was significantly lower ($p<0.001$) in the RA hyposalivary xerostomic (RA HSX) patients in comparison to the RA xerostomic patients (RA NSX). The salivary flow in both xerostomic rheumatoid arthritis groups was significantly lower in comparison to the healthy controls and RA non-xerostomic (RA NS) patients ($p<0.001$). The salivary flow in RA non-xerostomic (RA NS) patients was insignificantly higher in comparison to the healthy controls with $p=0.48$ (Fig. 1E).

No statistically significant group differences were found for age, laboratory markers, Schirmer I-test and stomatological values. In the RA xerostomic and RA non-xerostomic groups, no patients had a positive ANA, anti-SSA, or anti-SSB, whereas four RA hyposalivary xerostomic patients had a positive ANA.

There was no significant correlation between the CRP concentration, ESR, RF, WR, DMFT, GI, OHI-S indexes, salivary flow rate and the specific activity of HEX, HEX A, HEX B, or GLU in the saliva of any groups of the RA patients and healthy controls.

DISCUSSION

It was proven that salivary gland dysfunction during RA develops in two phases. The first phase of development of the disease is characterized by activation of epithelial cells covering acinar and ductal cells of the salivary glands [10,12]; during this phase of the disease normosalivation is observed (>0.1 mL/min). A key feature of the next phase is chronic inflammation with the influx of lymphocytes, antibody production and destruction of the salivary glands, which clini-

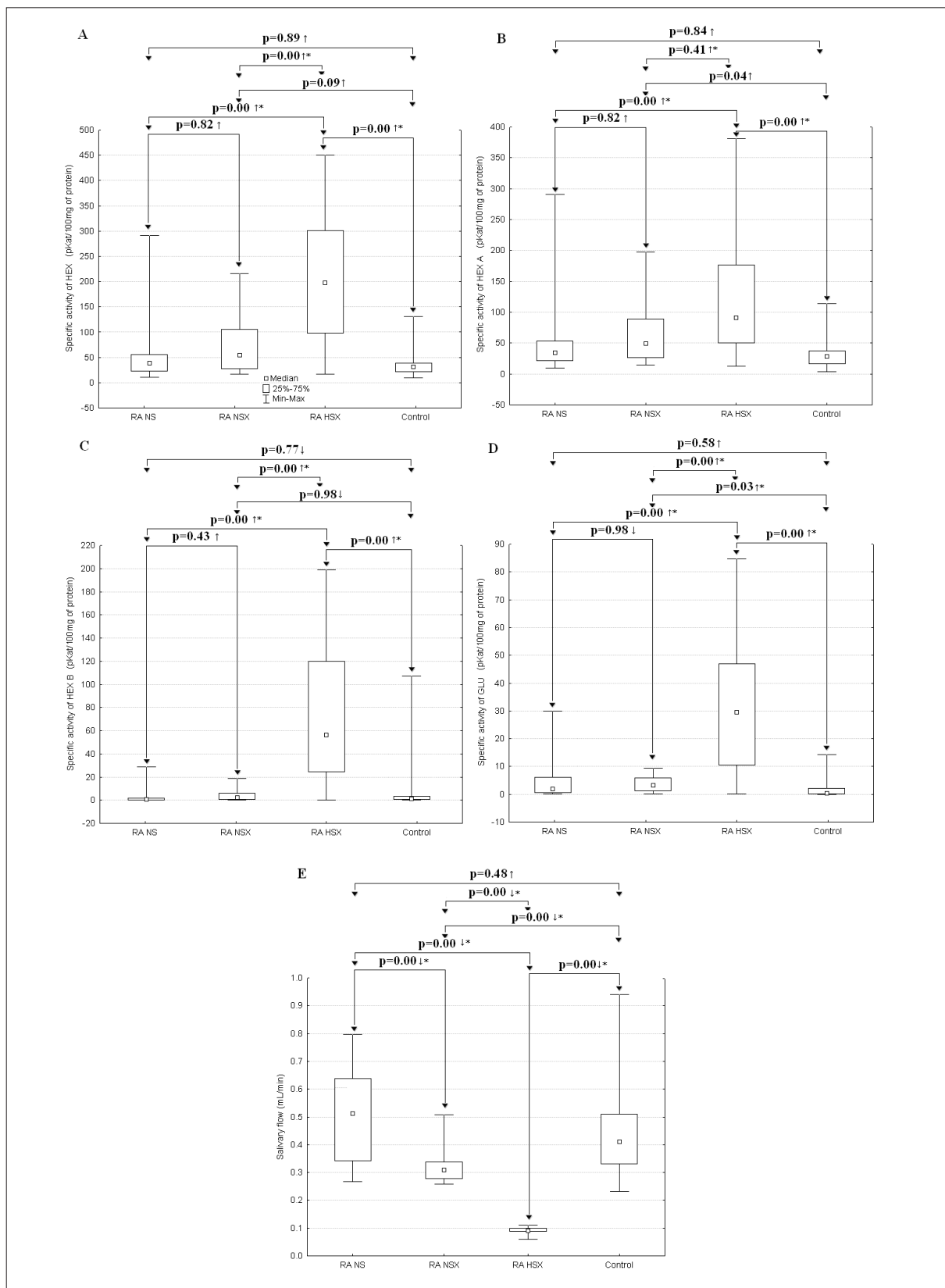


Fig. 1. Specific activity (pKat/100 mg of proteins) of A - HEX, B - HEX A, C - HEX B, D - GLU; E - salivary flow (mL/min) N-acetyl-β-hexosaminidase: HEX; isoenzymes of N-acetyl-β-hexosaminidase: HEX A, HEX B; β-glucuronidase: GLU; RA NS – non-xerostomic rheumatoid arthritis patients; RA NSX – xerostomic rheumatoid arthritis patients, RA HSX – xerostomic hyposalivary rheumatoid arthritis patients, C – healthy controls; significant increase ↑*, significant decrease ↓*, insignificant increase ↑, insignificant decrease ↓; p < 0.05 (Kruskal-Wallis test)

cally is observed as hyposalivation (≤ 0.1 mL/min). It was shown that the salivary gland impairment in the course of rheumatoid arthritis is accompanied by xerostomia [11, 24]. It was also shown that xerostomia is not only a subjective symptom of oral dryness, but also an indicator of early salivary gland dysfunction [30]. However, xerostomia is only a subjective symptom; therefore other more reliable indicators of the incipient phase of the disease are sought. As exoglycosidases are potential markers of salivary gland damage, we decided to evaluate the possibility of making use of HEX, its isoenzymes and GLU activity in the detection of early onset of salivary gland impairment in RA. For this purpose a group of RA patients with xerostomia (latent salivary gland dysfunction) was compared with a group of RA patients with xerostomia and hyposalivation (clinically symptomatic salivary gland dysfunction), a group of RA patients with normal salivary flow and without xerostomia (normal salivary gland function) and a reference group, without RA or xerostomia and with normosalivation.

It is worthy of note that in RA non-xerostomic patients (RA NS), the specific activities of lysosomal exoglycosidases were approximated to the data in the healthy controls, which suggests lack of changes in the catabolism of glycoconjugates in their salivary gland and it may confirm normal salivary gland function. It has been proven that an increase in the activity of lysosomal enzymes, including exoglycosidases, causes an increase in extracellular matrix degradation, which disturbs the communication between acinar cells of the salivary glands and nerve terminals and leads to the reduction of the secreted saliva [9]. It is also highly likely that the higher activity of the exoglycosidases intensified lysosomal degradation of glycoconjugates of the salivary gland tissue, which consequently leads to a reduction of the active secretory surface of the glands and a reduced volume of saliva secreted [26]. In the present paper we showed that xerostomia in RA NSX patients was accompanied by a significant reduction of salivary flow and a significant increase in the specific activity of GLU and HEX A vs. healthy controls; however, it appeared higher than 0.1 mL/min. We think that this significantly decreased secretion of saliva may be evidence of the beginning of the dysfunction of the secretory cells of the salivary glands, which may be caused partly by the mentioned exoglycosidases. A significant increase in the specific activity of HEX, HEX B and GLU in the saliva of RA xerostomic hyposalivary (RA HSX) patients as compared to the RA xerostomic (RA NSX) group may be evidence of an intensification of the glandular tissue damage in the more advanced phase of salivary gland destruction in RA, which results in a reduction of saliva ≤ 0.1 mL/min. In the present study we did not demonstrate the existence of a correlation between exoglycosidase activities and the amount of saliva, which does not exclude the involvement of exoglycosidases in the destruction of the salivary glands in the course of RA; it may be due to the small number of patients in the study groups and requires confirmation in a larger number of patients.

There are no known exact mechanisms of increases in the specific activity of salivary lysosomal exoglycosidases in both RA xerostomic groups (RA NSX) (RA HSX). It is known that salivary exoglycosidases derive from the glandular cells of salivary glands, bacteria, multinuclear leukocytes and macrophages of the crevicular gingival fluid during gingival inflammation [14]. We did not find any relationship between hygiene of the oral cavity or gingiva status and the activity of salivary exoglycosidases. There were no relations between the activity of general inflammation and the specific activity of salivary exoglycosidases. Lack of correlation between the activity of salivary lysosomal exoglycosidases and parameters of the general and local inflammatory states suggests that lysosomal exoglycosidases may be a marker of damage to the tissue of glandular salivary glands, independent of the general inflammatory state. The observed significant increase in the specific activity of GLU in the saliva of RA NSX patients vs. healthy controls may result from the aforementioned activation of epithelial cells of the salivary glands in the initial phase of salivary gland damage and subsequent influx of neutrophils, since the salivary GLU is accepted as an indicator of neutrophil influx and a marker for primary granule release by neutrophils [8]. Chronic inflammation, in the second phase of salivary gland damage, may cause a further significant increase in the specific activity of GLU in the saliva of RA HSX (vs. RA NSX) patients. Although neutrophils are a feature of acute inflammation, chronic inflammation by the action of inflammatory mediators (e.g. IL-1, IL-6, TNF released by activated endothelial cells and T lymphocytes present in the glandular infiltration) inhibits neutrophil apoptosis and extends their life [16].

A significant increase of the specific activity of HEX A in the saliva of RA NSX patients suggests increase in the secretory activity of the salivary gland cells, as the activity of HEX A reflects the secretory activity of the cell [31]. The observed increase in HEX A may be associated with activation of the epithelial cells in the early stages of salivary gland damage [27, 31]. It can also be a good indicator of activation of these cells; however, it requires confirmation in a larger number of patients.

It is worth noting that the increased specific activity of lysosomal exoglycosidases in the secreted saliva may cause accelerated degradation of glycoproteins of the pellicle and glycoproteins present in the saliva and result in disturbance of the oral cavity homeostasis, as well as in the development of many oral diseases, including xerostomia, so in this respect our results are also important for clinicians [1].

One limitation of our study is that there were only 16 patients in each study group; however, the results presented may confirm that determination of exoglycosidases may be a useful tool for the diagnosis of early stages of salivary gland involvement in RA, before development of hyposalivation.

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