Concentration of Nitric Oxide in Saliva of Patients with Rheumatoid Arthritis

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Abstract

Background: Nitric Oxide (NO) participation is recognized in numerous physiological and pathological processes. Rheumatoid arthritis (RA) is an inflammatory autoimmune disease involving joints and other systems including salivary glands. The role of NO in pathogenesis of development of RA is still unknown.

Aim & Objectives: We investigated NO concentration in saliva of 63 patients with RA and in 31 healthy control individuals. The aim of the study was also to investigate the correlation between saliva NO concentration and disease activity score (DAS28) in RA patients and to determine whether the statistically significant difference in saliva NO concentrations exists between RA patients with different stages of disease activity.

Methods: Patients with RA in this cross-sectional study have been divided, based on the stage of disease activity evaluated by DAS28score, into three subgroups: low disease activity (n=19), moderate disease activity (n=19) and high disease activity (n=25).
NO concentration was determined by measuring nitrite concentration by Griess reaction. Conversion of nitrate (NO^{-3}) to nitrite (NO^{-2}) was done with elementary zinc. Absorbance was measured at 546 nm with the use of spectrophotometer.

Results: Results have shown that saliva NO concentration in patients with RA (33,2 ± 4,8 μmol/dm^3) was statistically significant higher compared to saliva NO concentration in healthy controls (22,6 ± 2,3 μmol/dm^3; $p<0,05$). We found statistically significant negative linear correlation between saliva NO concentration and DAS28 score in RA patients ($r= -0,256$; $p<0,05$). Statistically significant difference between saliva NO concentration in RA patients with different stages of disease activity was not found.

Conclusion: This study indicates that NO may play an important role in the pathogenesis of RA and saliva NO concentration probably can be used as useful biochemical marker for evaluation the disease activity of patients with RA.

Key words: rheumatoid arthritis **(RA)**, disease activity score **(DAS28)**, nitric oxide **(NO)**

Introduction

Nitric oxide (NO) is a gas, messenger molecule with numerous physiological and pathological functions in human organism (1).

NO is synthesized from L-arginine in many tissues and cell types by the three established isoform of enzyme nitric oxide synthase (NOS): endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible (iNOS) (2).

NO is highly labile molecule and has a very short half-life of only a few seconds in plasma. Because of that, plasma concentration of his stable metabolites, nitrate (NO^{-3}) and nitrite (NO^{-2}), are often used as a marker of NO production and NOS enzyme activity. The effects of NO depend on its concentration (3). Constitutive isoforms of NOS (eNOS and nNOS) are dependent on intracellular concentration of calcium and calmodulin. In basal, physiological conditions low, transient levels of NO is continually being produced by this isoforms. NO produced by constitutive isoforms has a central role in vascular relaxation, neurotransmission, platelet aggregation, mitochondrial functions and immune regulation (4-6). The expression of iNOS is induced by inflammatory mediators. Large amounts of NO, generated primarily by iNOS, may contribute to NO-dependent tissue injury and tissue toxicity in a variety of human diseases processes.

There is a large number of evidence that speak of the fact that NO is involved in immune, inflammatory and autoimmune basis of arthritis and other disorders of muscular and skeletal systems (7). Rheumatoid arthritis (RA) is the most common system disease of connective tissue. 1-2% of the population worldwide is suffering from RA (8). The disease may appear at any age, but it is most common among aged from 40 to 70 years and its incidence increases with age (9).

RA is a chronic inflammatory disease that primary attacks the joints, causing inflammation, pain and swelling. Etiology and pathogenesis of RA is still to a great extent unknown and unclear.

Several studies have documented evidence for over production of NO in patients with RA what can be important in the pathogenesis of RA. Farrell *et al*. (10) reported increased concentration of NO^{-2} in synovial fluids and serum from patients with RA and osteoarthritis (OA). Predominant source of NO in RA are inflamed joint. Different cell types are capable of generating NO in the inflamed synovium, including osteoblasts, osteoclasts, macrophages, fibroblasts, neutrophils and endothelial cells (11). Synoviocytic fibroblasts seem to be the main source of NO in rheumatoid synovium (12).

Inflammatory state besides joints, involve several other organs, including skin, lungs, blood vessels, eyes and salivary glands (13).

Although salivary gland involvement in RA has been known for a long time, it did not draw much attention from researchers.

Morphological studies revealed that the minor salivary glands of RA patients were highly infiltrated with lymphocytes and that the alterations also included fibrosis, acinar atrophy and lymphoplasma cell sialadenitis (14,15).
In spite of the relatively large amount of accumulated evidence that the salivary glands are profoundly affected in RA, there is no available study analysing the concentration of NO of the secreted saliva of these patients.
The only authors who examined the concentration of NO, apart from serum and urine, in the saliva of patients with RA were Weinberg *et al* (16).
These authors have demonstrated reduced flow of stimulated and unstimulated saliva in patients with RA compared to healthy subjects.
From reviewing the existing studies and literature we did not find data about concentration of NO in saliva of RA patients with different stages of disease activity and data about relationship between saliva NO concentrations and stages of disease activity in patients with RA.

Methodology

Subjects

This cross-sectional study included 63 (59 females and 4 males) patients diagnosed with RA, according to the 1988. revised American College of Rheumatology (ACR) criteria (17) who attended the Clinic of Heart and Rheumatic Diseases at the Clinical Center University of Sarajevo during 2011. Patients with RA, based on the stage of disease activity, have been divided into three subgroups: low stage of disease activity (DAS28 $\leq$ 3.2; n=19); moderate stage of disease activity (3.2 < DAS28 $\leq$ 5.1; n=19); high stage of disease activity (DAS28 > 5.1; n=25).
Disease activity in RA patients was carried out by the disease activity score (DAS28) using erythrocyte sedimentation rate (ESR). The formula used in this study was the DAS28 with four variables:
DAS28= $0.56x\sqrt{(TEN28)} + 0.28x\sqrt{(SW28)} + 0.70xLn(ESR) + 0.014x(GH)$.
(TEN28: 28 joint count for tenderness, SW28:28 joint count for swelling, Ln(ESR): the natural logarithm of Westergren's ESR, GH: general health or patient's global assessment of disease activity on the visual analog scale (VAS) of 100 mm). DAS28 was calculated by experienced physician.
The control group consisted 31 (27 females and 4 males) age- and gender-matched apparently healthy individuals. Both groups were aged between 29 and 83 years.
Exclusion criteria included other rheumatic diseases or any local or systemic illness that is known to be able to have some influence on the concentration of NO in saliva.
Detailed history about patients was taking using specially prepared questionnaires condition, including disease duration, morning stiffness and drug history. All patients underwent thorough clinical examination with special attention to articular examination.
Approval for the study was obtained from the local ethics committee. All procedures on human subjects were performed in the accordance with Helsinki Declaration of 1975. Informed consent was obtained from subjects and caregivers upon careful explanation of the study procedure.

Methods

The NO level in the saliva was determined by measuring NO^{-2} concentrations, a stable metabolic product of NO with oxygen. Saliva samples from the patients and controls were

taken after an overnight fast and after rinsing the mouth with running water for 60 seconds to reduce bacterial contamination. To remove remaining debris and microorganisma, we centrifuged saliva sample 5 minutes at 3000 g. After that, we removed proteins from saliva samples so that one milliliter of sample is added 0,05 ml 30% solution of zinc sulfate. After several minutes, the sample is centrifuged 10 minutes at 700 g and separated supernatant is stored at -20^{0}C to determine the concentration of NO^{-2}.
Conversion of NO^{-3} to NO^{-2} was done with elementary zinc. NO^{-2} concentration in saliva was determined colorimetrically using freshly prepared Griess reagents (18).
After 10 minutes of mixing the vibrator, at room temperature, it was measured by light absorption (optical density) spectrophotometer with a filter of 546 nm. The concentration of NO^{-2} was determined by a standard curve with known concentration of sodium nitrite (from 1,56 to 100 nm). As the blank distilled water, to which Griess reagent was added, is used. The results were expressed as μmol/dm^3.
Determination of NO concentration in saliva of patients and control group was done at the Department of Physiology on Medical Faculty in Sarajevo.

Statistical analysis
All analysis was performed with Statistical Package for the Social Sciences (SPSS) version 17 for Windows. Data are presented as mean value (X) and standard error of mean (SEM). Data were statistically analysed with the non-parametric Mann-Whitney U test. Correlation between variables was assessed by Sperman's rank correlation coefficient. Statistical significance was set at $p<0.05$.

Results

The mean age of the healthy subjects in control group ± standard errors (n=31; 4 males, 27 females) was 49,68 ± 1,42years; whereas for the RAgroup was 53,38 ± 1,45 (n=63; 4 males, 59 females) **(Table 1)**.
The results show that the values (X±SEM) of the concentration of NO in the saliva of patients with RA (33,2 ± 4,8 μmol/l) were higher by 46,9% compared to the values (X±SEM) specified in the control group of the healty subjects (22,6 ± 2,3 μmol/l). This higher NO concentration in saliva of patients with RA was statistically significant compared to the control group ($p<0,05$) (**Figure 1)**.
Weak negative linear correlation was notes between DAS28 score and saliva concentration of NO in patients with RA ($r= -0,256$; $p<0,05$) (**Figure 2)**.
Values (X±SEM) of the concentration of NO in saliva of RA patients with moderate disease activity (24,9 ± 3,9 μmol/l) were lower by 102% compared to the values (X±SEM) of saliva NO concentration specified in the group of patients with low disease activity (50,3 ± 12,6 μmol/l).
The concentration values(X±SEM) of NO in the saliva of RA patients with high disease activity (26,5 ± 6,1 μmol/l) were lower by 89,81% compared to the values (X±SEM) of saliva NO concentration specified in the group of patients with low disease activity (50,3 ± 12,6 μmol/l), but it was higher by 6,04% compared to the values (X±SEM) of saliva NO concentration specified in the group of patients with moderate disease activity (24,9 ± 3,9 μmol/l)
However, saliva NO concentration (X±SEM) was not statistically significantly different between RA patients with different stages of disease activity ($p>0,05$) (**Figure 3)**.

DISCUSSION

This study evaluated that the concentration of NO in the saliva of patients with RA was statistically significantly higher than the concentration of NO in the saliva of healthy subjects ($p<0,05$). Our findings of increased NO concentration in the saliva of RA patients are not in the accordance with results of Weinberg *et al.* (16). These authors reported that basal and stimulated salivary nitrate concentrations were not different between healthy controls and patients with RA.

The common extra-articular manifestation of RA is a dysfunction of lacrimal and salivary glands, which gradually leads to the development of sicca syndrome, respectively secondary Sjögren's syndrome (sSS) in these patients. According to Weinberg *et al*. (16), the prevalence of sicca syndrome in patients with RA ranges from 25% to 65%. Research has shown that the sSS in patients with RA occurs more frequently than in patients with other diseases of muscle, bone and connective tissue. Grevers *et al.* (15) reported that 20% of the RA patients in their studies also suffered from sSS.

However, the association of RA and sSS is still not fully understood. Uhlig *et al.* (19) found a high prevalence of ocular and/or oral sicca syndrome in patients with RA and positive association between hyposecretion of salivary glands and disease activity that was not related with duration of disease or with the number of joints affected by disease.

In our study, we did not investigate the salivary gland flow in patients with RA. However, using a questionnaire we received information that 42.9% of patients included in our study had current subjective feeling of reduced amounts of saliva compared to the previous period.

Healthy subjects did not have feeling of dry mouth and reduced saliva secretion. The difference in prevalence of xerostomia symptoms between this two groups was statistically significant ($p<0.0001$). Obtained results indicate presence of xerostomia in RA patients involved in our study.

The significant reduction in salivary gland flow in the RA patients points to the salivary glands as major target organ of RA.

Given that the literature has little information about the results of research on NO concentration in saliva of patients with RA, we find it difficult to compare our results with the results of other studies. However, considering the fact that RA is a systemic disease of connective tissue that can affect exocrine glands, we compared our results with results of studies that examined the concentration of NO in the saliva of patients with primary Sjögren's syndrome (pSS). Recent studies show hypofunction of salivary glands in patients with pSS, but higher concentrations of nitrite and iNOS in the salivary gland acini in these patients (20).

Takeda *et al.* (21) found increased salivary NO concentration with accompanying xerostomia in patients who were exposed to X-ray radiation.

The causes of increased salivary NO concentration and xerostomia of RA patients in our study may be different. One of the reason overall increase in salivary NO concentration in RA patients may result from the general increase in serum NO concentration, because to a large extent plasma composition is reflected in saliva composition (22).

It may also reflect a specific response of the salivary glands to the RA. We assume that an increase in NO concentration in the saliva of patients with RA can occur due to inflammatory processes acinic and ductal epithelial cells of salivary glands and, consequently, increased iNOS activity of inflamed tissue, under whose influence the NO produced in higher concentrations. NO can be regarded as a potential cause for decreased salivary gland function in RA patients, that leads to apoptosis of gland acini, and to the decreased production of saliva.

There are only a few studies about the relationship between disease activity and concentration of NO in patients with RA.
Ueki *et al.* showed that serum NO concentration in RA patients correlated with indices of disease activity and inflammatory cytokines, whereas ESR and the Landsbury index revealed no significant relationship (23).
Similar to the above findings, Onur *et al*. (24) demonstrated elevated serum concentration of nitrate in RA patients and a significant correlation between serum nitrate concentrations with a number of tender joints, swollen joints, Ritchie articular index, DAS score and C-reactive protein level.
In contrast, examining the relationship between disease activity and concentration of NO in saliva of patients with RA we found a statistically significant, negative, linear correlation ($r = -0,256$; $p<0,05$) between these two variables. According to this, examining difference in concentration of NO in saliva of RA patients with different stages of disease activity, we found that patients with low stage of disease activity (DAS28 $\leq$ 3.2) had 100% higher NO concentration in saliva compared to the patients with moderate stage of disease activity (3.2 < DAS28 < 5.1) and for about 90% higher NO concentration compared to the patients with high disease activity (DAS28 > 5.1). However, these differences were not statistically significant.
This result probably indicates that increased disease activity score in patients with RA decrease saliva concentration of NO in these patients. It is possible that this relationship between disease activity and concentration of NO in saliva of patients with RA may reflect one of many abnormalities in immune regulation.

Conclusion

Increased Nitric Oxide (NO) production plays an important role in the pathogenesis of rheumatoid arthritis (RA). These findings may have implications for the diagnosis of RA and led to improvements in measures of disease activity. Determination of NO concentration in the saliva of patients with RA can be used in assessing disease activity.
Future studies are needed to determine the exact role of salivary NO in assessing of disease activity in RA patients.

List of Abbreviations:
NO- nitric oxide
RA - rheumatoid arthritis
DAS28 - Disease activity score
NO^{-3} - nitrate
NO^{-2} - nitrite
NOS - nitric oxide synthase
eNOS - endothelial nitric oxide synthase
nNOS - neuronal nitric oxide synthase
iNOS - inducible nitric oxide synthase
OA - osteoarthritis
ACR - American College of Rheumatology
ESR - erythrocyte sedimentation rate
TEN28 - 28 joint count for tenderness,
SW28 - 28 joint count for swelling,
Ln(ESR) - the natural logarithm of Westergren's erythrocyte sedimentation rate

GH- general health or patient's global assessment of disease activity on the visual analog scale of 100 mm
VAS- visual analog scale
sSS - secondary Sjögren's syndrome
pSS - primary Sjögren's syndrome

Conflict of Interest: Authors do not have any commercial affiliations, or potential conflicts of interest associated with this work submitted for publication.

Authors' Contributions: All authors of this research paper have directly participated in the planning, execution, and analysis of this study.

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Table 1: Age and gender distributions of healthy subjects and RA patients

	Total	Male	Female	Age (yr) mean±S.E.M.
Control group	31	4	27	49,68 ± 1,42
RA (total)	63	4	59	53,38 ± 1,45

Control group - healthy subjects
RA - group of patients with RA

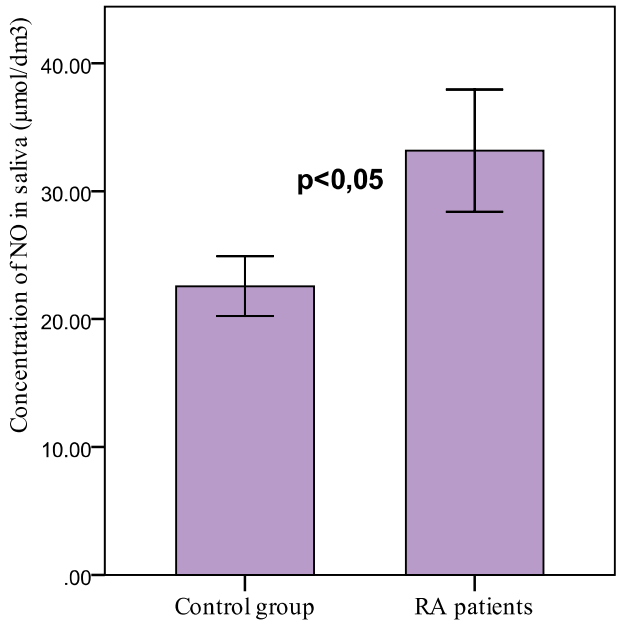

Figure 1. NO concentrations in saliva in control group and in the group of patients with RA: Presented mean values (X±SEM) concentration of NO in saliva (µmol/dm^3) in the control group of healthy subjects and in the group of patients with RA.

Control group- healthy subjects (n=31; 27 females and 4 males);
RA patients - group of patients with reumatoid arthritis (n=63; 59 females and 4 males)
p< - significant difference

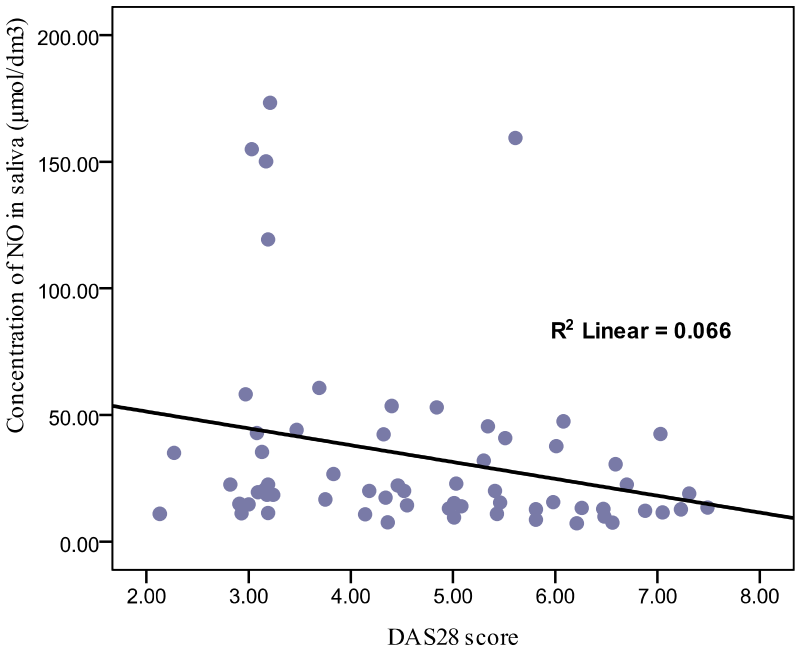

Figure 2. Linear correlation between DAS28 score and saliva concentration of NO in RA patients (n=63): Presented weak negative linear correlation between DAS28 score and saliva

concentration of NO (μmol/dm^3) in the group of patients with RA. The Spearman rang correlation coeficient (r) was -0.256 (p<0,05).

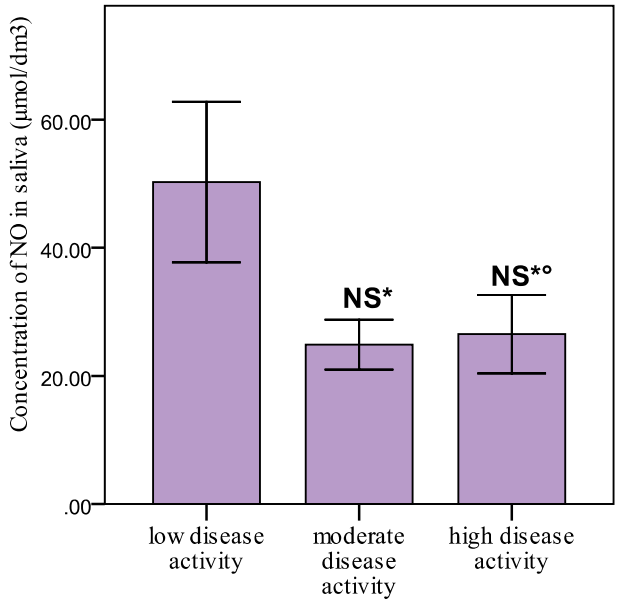

Figure 3. NO concentrations in saliva of RA patients with different stages of disease activity: Presented mean values (X±SEM) concentration of NO in saliva (μmol/dm^3) RA patients with different stages of disease activity.

Low disease activity (DAS28 < 3.2; n=19);
Moderate disease activity (3.2 < DAS28 < 5.1; n=19);
High disease activity (DAS28 > 5.1; n=25).
NS – not significant.
* – versus patients with low disease activity
° – versus patients with moderate disease activity