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ORIGINAL ARTICLE

Telomere dysfunction-related serological markers and oxidative stress markers in rheumatoid arthritis patients: correlation with diseases activity

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Abstract

Rheumatoid arthritis (RA) is an inflammatory autoimmune polyarthritis with progressive destruction of the synovial joints associated with systemic manifestations. RA is characterized by infiltration of the synovial joints with inflammatory immune cells with premature immunosenescence. Shorter telomere length in the peripheral blood cells and increase in the oxidative stress have been detected in patients with RA. The aim of the present study was to study the association of markers of telomere shortening and oxidative stress with RA disease activity. Sixty-one RA patients and 15 healthy controls were enrolled in the study. Demographic data, clinical examination, and disease activity status were evaluated for the RA patients. Serum levels of chitinase and NAG (telomere markers) were determined by biochemical reactions using colloidal chitin and NAG as substrates, respectively. Nitric oxide and superoxide dismutase (oxidative stress markers) were determined colometrically and spectrophotometrically, respectively, in the sera of RA patients and controls. Results were correlated with disease activity. Indices of telomere shortening and oxidative markers were significantly higher in RA patients compared to controls. These indices were correlated with signs of disease activity (including number of swollen and tender joints, DAS-28, and inflammatory markers). Rheumatoid arthritis is a disease in which markers of telomere shortening and elevated oxidant stress correlate with disease activity.

Keywords Disease activity · Oxidative stress · Rheumatoid arthritis · Telomere dysfunction

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory polyarthritis with progressive destruction of the synovial joints associated with systemic manifestations. RA significantly decreases the patients' functional capacity, increases the morbidity and mortality rates, and results in significant costs for the health care system [1, 2]. During the active stage of the disease, there is severe systemic inflammation and hyperplasia of the synovium of the joints with cellular infiltration [3]. One of the pathognomonic features of RA is the accelerated aging of the immune system (immunosenescence) cells

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[4] that favors the disruption of self-tolerance which is a fundamental process in the onset of RA.

Telomere erosion, which is a hallmark of immunosenescence, is present in lymphoid (naïve and memory T cells) and myeloid (granulocytes) cells in RA [5]. Telomeric repeats decrease by 30 to 200 base pair (bp) with each successive cell division. While the set point of telomeric length is genetically determined, external factors affect telomere attrition and are not constant, but are affected by age, inflammation, oxidative stress, antioxidative defenses, and cell turnover [2, 6]. In RA, there is an increase in the oxidative stress due to production of reactive oxygen intermediates by polymorphonuclear leukocyte and lymphocyte. Moreover, lymphocytes in RA showed cellular hyperactivity to the toxic effects of hydrogen peroxide [3].These factors favor telomeric shortening and, in fact, telomeric shortening is observed in RA patients compared to age-matched controls [7].

Oxidative stress can be measured by measuring lipid peroxides (LPER) and nitric oxide (NO) and the antioxidant enzyme, superoxide dismutase (SOD). A set of biomarkers, namely Cathleen-related antimicrobial peptide (CRAMP), Stathmin, elongation factor alpha (EG-1a), chitinase, and n-acetyl-glucosaminidase (NAG) have been identified as indices of telomere dysfunction [8, 9].

In the present study, we measured two biomarkers of telomere dysfunction (chitinase and NAG) and three measures of oxidative stress (LPER, NO, SOD) in RA patients and examined the relationship of these markers to RA disease activity.

Patients and methods

Study population

Sixty-one ($n = 61$) consecutive RA patients recruited from Physical Medicine, Rehabilitation and Rheumatology department outpatient clinic, at Assiut University Hospitals were invited to participate in this cross-sectional study. Patients were eligible to participate if they were ≥ 18 years and diagnosed according to 2010 American College of Rheumatology (ACR) classification criteria for RA [10] with the use of stable medication in the last 3 months. Gender-matched healthy participants ($n = 15$) were recruited from the university and hospital workers and served as the control group. The exclusion criteria for patients were: Crohn's disease, ulcerative colitis, malnutrition, hyperparathyroidism, hyperthyroidism, renal and hepatic diseases, and any limitation of physical activity or medications that might affect bone metabolism or the endocrine system (e.g., thyroxin, anticonvulsants, hormone, or vitamin D replacement therapy). Before entering the study, participants voluntarily signed the informed consent form which had been approved by the Faculty Ethics Committee.

Disease variables and activity assessment

Sociodemographic data including age and gender were obtained from all the study participants. For the patients' group, clinical characteristics included disease duration, extra-articular involvement, presence of comorbidities (e.g., hypertension and diabetes mellitus (DM)), and family history of RA or autoimmune diseases. Past or current medications were ascertained, including non-steroidal anti-inflammatory drugs (NSAIDs), any disease modifying anti-rheumatic drugs (DMARDs) such as methotrexate (MTX), hydroxychloroquine, leflunamide, sulfasalazine (SSZ), cortico-steroids, or biological DMARDs such as abatacept, tocilizumab, rituximab, or TNF inhibitors. Disease activity status was assessed using the Disease Activity Score including 28 joint counts (DAS-28-ESR). The Components of DAS-28-ESR were swollen and tender joint counts (each 0–28), patient-assessed global score (0–100), and erythrocyte sedimentation rate (ESR). High disease activity was defined as a DAS28-ESR > 5.1, moderate disease activity was defined as a 3.2 < DAS28-ESR ≤ 5.1, low disease activity was defined as a 2.6 ≤ DAS28-ESR ≤ 3.2 and remission as a DAS28-ESR < 2.60 [11, 12].

Laboratory assessments

Determination of the ESR, RF, complete blood counts, fasting blood glucose, and hemoglobin A_1C were conducted by routine laboratory methods. N-acetyl glucosaminidase (NAG) activity was determined by colorimetric test according to Skrhaet al. [13] by using N-acetyl-p-nitrophenyl—β-glucosaminidase as a substrate. Activity of chitinase was evaluated using colloidal chitin as a substrate. Colloidal chitin 1% (*w*/*v*) was prepared using the method described by Lee et al. [14]: 1 g chitin powder was added slowly to 20 ml of concentrated HCL and left at 4 °C overnight with vigorous stirring. The mixture was added to 200 ml of ice-cold 95% ethanol with rapid stirring and kept overnight at 4 °C. The precipitate was then collected by centrifugation at 5000×*g* for 20 min. at 4 °C and washed with sterile distilled water until the colloidal chitin became neutral (pH 7.0). The final volume was then raised to 100 ml by adding 50 mM pH 6.5 sodium phosphate buffer. The reaction mixture consisted of 1 ml % (*w*/*v*) colloidal chitin and 1 ml enzyme solution. After incubation at 60 °C for 45 min, the reaction was stopped by heating in boiling water for 20 min. Then the mixture was centrifuged immediately at 10,000×*g* for 5 min. The supernatant was used for determination of N-acetyl-D-glucosamine released as described by Reissig et al. [15]. One unit (U) of chitinase activity was defined as amount of the enzyme required to release 1 μmol N-acetyl-D-glucosamine per minute. The level of nitric oxide (NO) was measured colometrically as total nitrite + nitrate as described previously [16]. The activity of super-

oxide dismutase (SOD) was determined spectrophotometrically according to the method described before [17]. LPER was measured by the method described in previous work [18].

Statistical analysis

Data were presented as number and percentage, or mean ± SD as appropriate. Statistical significance was assessed via $\chi 2$ for categorical variables and Student's t test for continuous variables. No compensation for repeated measures was done. Multiple regression analysis was used to test the association between our biomarkers and RA features. Data were analyzed by Statistical Package for Social Science (SPSS) version 20.0 and p values < 0.05 were considered statistically significant.

Results

General characteristics of RA patients and controls

RA patients aged 43.89 ± 11.79 (range18–69) years and were 57 females (93.4%) and 4 males (6.6%); the control group was aged 48.93 ± 11.35 (28–65) years and were 6 females (40%) and 9 males (60%). Other clinical and laboratory data of the RA patients are shown in Table 1.

Telemetric shortening, oxidative stress, and antioxidants markers in RA and controls

Levels of telomere shortening, chitinase, (120.96 ± 29.44 versus 57.78 ± 12.02 u/mL, $p = 0.001$) and NAG (13.42 ± 3.07 versus 6.53 ± 1.08mIU/ml, $p < 0.001$) were significantly

Table 1 Clinical and laboratory characteristics of RA patients (No. = 61)

Item		No.	%
Clinical findings	Dis. duration (year)	7.14 ± 5.0 (range 1–22)	
	MS/h	1.07 ± 0.6 (range 0.17–2)	
	No. of swollen joints	2.52 ± 2.95 (range 0–16)	
	No. of tender joints	7.57 ± 6.4 (range 0–22)	
	Global score	17.95 ± 16.26 (range 0–60)	
	DAS-28-ESR	4.36 ± 1.2 (range 1.63–6.9)	
Disease activity (DAS-28-ESR) categories	Remission	5	8.2
	Low	7	11.5
	Moderate	31	51
	High	18	29.5
HTN	Present	8	13
DM	Present	3	5
Current medications	MTX	24	39
	Hydroxychloroquine	51	84
	Leflunomide	30	49
	SSZ	7	11.5
	NSAIDs	27	44
	Steroids	20	33
Past medication	MTX	28	46
	Hydroxychloroquine	4	6.5
	Leflunomide	6	9.8
	SSZ	1	1.6
	NSAIDs	2	3.3
	Steroids	14	23
	Azathioprine	1	1.6
Laboratory data	ESR (Mm/h)	36.8 ± 18.24 (range 3–80)	
	WBCs	6.14 ± 2.8 × 10^9/L (range 2.8–18.4)	
	HB level (g/dl)	12.45 ± 1.6 (range 8.1–15.7)	
	Platelet count	302.43 ± 96.9 × 10^9/L (range 25–556)	

DM diabetes mellitus; *HB* hemoglobin; *HTN* hypertension; *ESR* erythrocyte sedimentation rate; *MS/h* morning stiffness per hour; *MTX* methotrexate; *NSAIDs* non-steroidal anti-inflammatory drugs; *RA* rheumatoid arthritis; *RF* rheumatoid factor; *SSZ* sulphasalazine; *WBCs* while blood cells

higher in RA patients compared to controls indicating increased telomere shortening. Measures of oxidative stress, LPER (11.19 ± 2.48 versus 4.47 ± 0.71 μmol/L, $p < 0.001$) and NO (14.76 ± 2.89 versus 8.61 ± 1.13 μmol/L, $p = 0.001$), were significantly higher in RA patients compared to controls and SOD levels were significantly lower in patients than controls (137.64 ± 37.23 versus 320.75 ± 66.06 u/L, $p = 0.01$) (Fig. 1).

Different disease activities showed significant differences in levels of telomere shortening and oxidative stress. For example as the DAS-28-ESR worsened from remission to high disease activity, markers of telomere shortening indicated progressive shortening (e.g., chitinase remission, 91 (18.9) to high DAS28-ESR 143.5 (26.3) ($p = < 0.001$). Likewise, markers of oxidative stress increased as disease activity increased (e.g., LEPR remission, 8.5 (1.5) to high disease activity: 12.8 (2.2, $p < = 0.001$) (Table 2).

Association of telometric shortening, oxidative stress, and antioxidants markers with clinical and laboratory data of RA patients

In the multivariate analyses, swollen joints were associated with telomere shortening—chitinase (beta = 1.157; $p = 0.027$) and NAG (beta = 0.118; $p = 0.033$) and oxidative stress—LPER (beta = 0.081; $p = 0.014$), and NO levels (beta = 0.109; $p = .003$). Chitinase associated with DAS-28-ESR (beta = 7.027; $p = 0.01$) and ESR (beta = 0.505; $p < .001$).

Among the biomarkers, there were a number of associations: NAG levels correlated significantly with levels of chitinase (beta = 0.076, $p < 0.001$), LPER (beta = 0.700, $p < 0.002$), SOD (beta = − 0.012, $p = 0.048$), and NO (beta = 0.494, $p = 0.014$). LPER was significantly associated with NAG (beta = 0.252, $p = 0.002$) and NO (beta = 0.779; $p < .001$, respectively). SOD correlated negatively with NAG (beta = − 5.987, $p = 0.048$) and NO (beta = − 15.095;

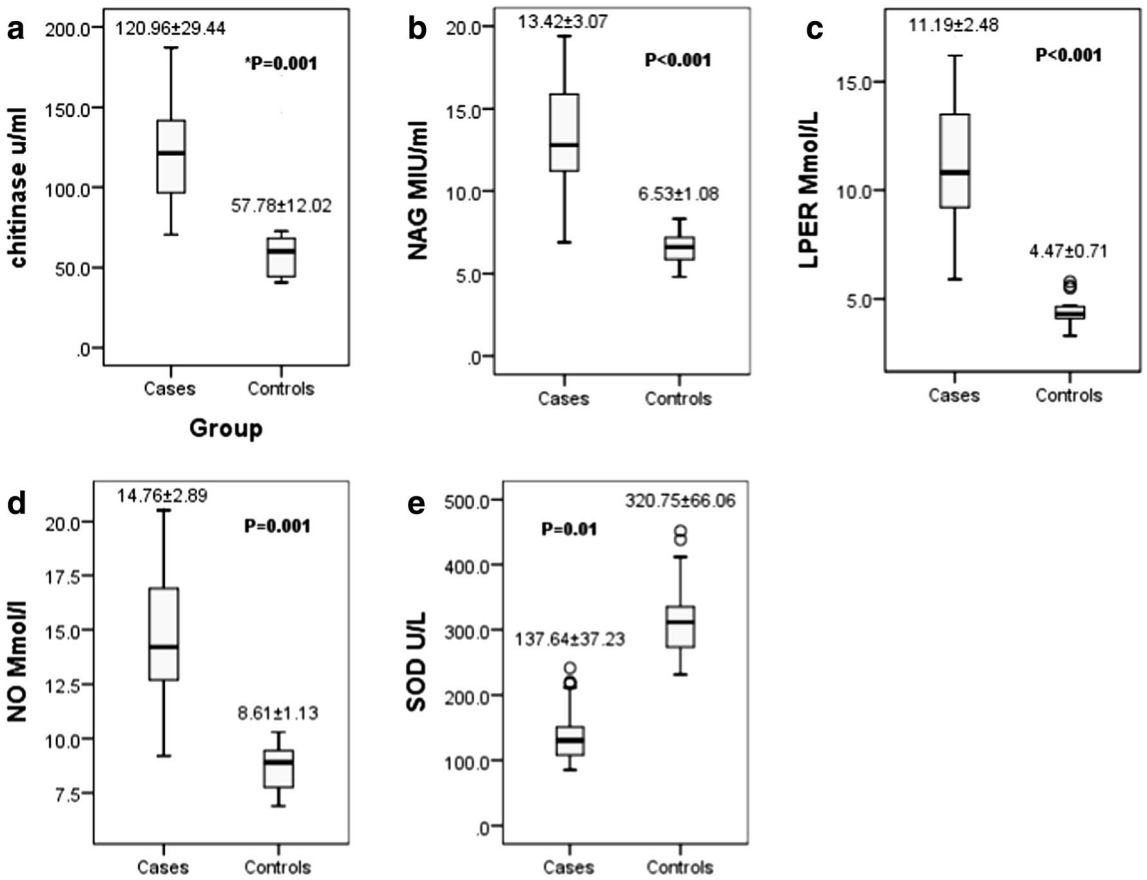

Fig. 1 A–E Levels of telomere dysfunction and oxidative stress biomarkers in rheumatoid arthritis cases and controls. Student's *t* test was performed and showed that levels of chitinase, NAG, LPER, and NO were significantly higher in RA patients compared to controls. However, SOD levels were significantly lower in patients than controls. Abbreviations: *LPER* lipid peroxides; *NAG* n-acetyl glucosaminidase; *NO* nitric oxide; *SOD* superoxide dismutase.

Table 2 Telomere shortening and oxidant markers in different disease activity categories in RA patient

DAS-28	Chitinase (u/ml)	NAG (mIU/ml)	LPER (μmol/L)	NO (μmol/L)	SOD (U/L)
Remission	91 ± 18.9 (78–124)	10 ± 1.8 (8.5–12.6)	8.5 ± 1.5 (7–10.6)	11.4 ± 1.9 (9.2–14)	182 ± 46.7 (135–242)
Low	108.4 ± 31.87 (70.6–151)	11.8 ± 3.5 (7–16)	10 ± 3 (6–13.8)	13.4 ± 2.8 (9.7–17)	160 ± 39 (129–220)
Moderate	115.5 ± 23.8 (82–169)	13 ± 2.4 (9–18)	11 ± 2 (8.5–15.2)	14.5 ± 2.5 (10.2–20)	137 ± 33 (90–218)
High	143.5 ± 26.3 (88–187)	15.7 ± 2.8 (9.5–19.4)	12.8 ± 2.2 (8–16.2)	16.6 ± 2.6 (11.6–20.5)	118 ± 27 (85–205)
p	< 0.001	< 0.001	0.001	< 0.001	0.001

DAS-28 disease activity score including 28 joint counts; *NAG* n-acetyl glucosaminidase; *NO* nitric oxide; *RA* rheumatoid arthritis; *SOD* superoxide dismutase

$p < .001$) (Table 3).In the univariate analysis, levels of SOD decreased significantly with increasing age ($p = 0.011$), although this was not found in multivariable analysis.

Discussion

RA is a chronic autoimmune disease characterized by immune system dysfunction with increased production of proinflammatory cytokines that play a major role in disease onset and progression [19]. Previous reports linked the process of chronic inflammation with telomere shortening [20, 21], which occurs after each cell division. Our study is supportive of the relationship between telomere dysfunction and oxidative stress, with multiple indicators showing that oxidative stress was associated with telomere shortening. Also, our study shows that progressive telomere shortening occurred in the presence of increasing disease activity and multivariable analysis showed an association with swollen joint counts and ESR.

The set point of telomere length is genetically determined but external factors including chronic stress and inflammation influence this length [2, 22] .With increased oxidative and inflammatory stress, there is increased Th17 T cell differentiation, although Th 17 cells appear relatively resistant to stress [6, 22] On the other hand, Th1 cells become senescent with oxidative stress and inflammation [6]. These effects of stress on the immune response can result in increased inflammation (i.e., relatively preserved Th17 function with decreasing Th1) and help explain the relationship we found between RA disease activity and telomere shortening. In addition, some investigators feel that telomere shortening due to excessive cell division in the face of inflammation is related to loss of self-tolerance which could contribute to the pathogenesis of RA [20, 23, 25, 29, 30].

Table 3 Multivariate regression analysis of telomere shortening and oxidative stress markers with the clinical and laboratory data in RA patients

Clinical and laboratory data	Chitinase (u/ml)		NAG (mIU/ml)		LPER (μmol/L)		NO (μmol/L)		SOD (U/L)	
No. of swollen joints	Beta	*p*	Beta	*p*	Beta	*p*	Beta	*p*	Beta	*p*
	1.157	*.027*	0.118	*.033*	0.081	*.014*	0.109	*.003*	− 0.135	0.913
No. of tender joints	0.609	0.088	0.038	0.323	0.003	0.888	0.001	0.967	− 0.974	0.241
DAS-28	7.027	*.010*	0.562	*0.052*	0.154	0.384	0.169	0.394	− 6.936	0.281
ESR	0.505	*<.001*	0.013	0.355	0.011	0.174	0.015	0.123	− 0.521	0.090
Platelet count	0.010	0.414	0.000	0.717	0.000	0.763	0.000	0.718	0.036	0.217
Chitinase (u/ml)			0.076	*<.001*	0.004	0.692	0.010	0.314	− 0.080	0.805
NAG (mIU/ml)	6.852	*<.001*			0.252	*.002*	0.226	*.014*	− 5.987	*.048*
LPER (μmol/L)01	0.882	0.692	0.700	*.002*			0.987	*<.001*	− 8.234	0.104
NO (μmol/L)01	1.981	0.314	0.494	*.014*	0.779	*<.001*			− 15.095	*<.001*
SOD (U/L)	− 0.015	0.805	− 0.012	*.048*	− 0.006	0.104	− 0.014	*<.001*		

DAS-28 disease activity score including 28 joint counts; *NAG* n-acetyl glucosaminidase; *NO* nitric oxide; *RA* rheumatoid arthritis; *SOD* superoxide dismutase

Values in italic are significant at $p < 0.05$

Our results showed that the mean levels of telomere-shortening markers (chitinase and NAG) were greater in RA patients than those in healthy controls, supporting others [3, 4, 8, 23–25].

Telomere shortening is also found in other autoimmune diseases such as SLE, Sjogren's syndrome, granulomatous disease, scleroderma, and axial spondyloarthitis [26–31]. In RA, the decrease in thymic output of T cells is inappropriate for the patients' age, actually matching healthy individuals who are 20–30 years older [32].

While our study showed an apparent effect of age on SOD in univariate analysis, this was not borne out in the multivariable analysis. Our data are supported by Steer et al. [33] who found that the telomere shortening was already present and different from normal at an early age and did not increase with age. More studies over extended periods are needed to explore this issue. Female sex hormones in RA patients did not affect the levels of telomere shortening or oxidative stress markers in our work which was not to be expected. Previous data revealed the protective role of female sex hormones against telomere erosion [34–36] and oxidative stress [34, 37–39].This discrepancy between our results and previous data might be attributed firstly to the small number of participants as a general. Secondly, menopausal RA females constituted only 21% of RA females and only four RA men were included in our work which limited the real comparing data of sexual effect on both telomere length and oxidative stress in RA.

Our data, utilizing DAS28-ESR as a measure of disease activity showed a relationship between disease activity and oxidative stress and demonstrated that at least one of the two telomere-shortening markers, chitinase, showed a significant correlation with DAS-28-ESR as well as components of the DAS-28-ESR separately. Yamanashi et al. [40] found some relationship with joint replacement, a potential marker for joint severity, but not necessarily activity. In SLE, a relationship was found with nephritis and in systemic sclerosis a measure of severity, interstitial lung disease, was associated with telomere shortening [26, 41].

Some studies in RA did not find this relationship [7, 42, 43]. Our differences may have been associated with methodological differences (e.g., measuring telomere shortening markers versus terminal restriction fragment lengths) associated with study design (cross-sectional design vs longitudinal design), or due to differences in patient clinical disease activity.

In addition to telomere shortening, cellular senescence can occur as a result of oxidative stress [44]. We found a significant increase in the level of LPER and NO in RA patients compared to controls, similar to others [45–49].

The markers of oxidative stress in the form of lipid peroxides, nitric oxide, and the antioxidant enzymes, SOD, were associated with selected clinical and demographic features of our RA patients, supporting the data of Kamanli et al. [50].

Our study had significant strengths. The patients exhibited a range of disease activities, including higher disease activity, which may have allowed us to see the relationship with disease activity not found by others. It used both validated markers of telomere shortening and oxidative stress, allowing us to examine both aspects of cellular senescence and thus enabling us to examine correlations of disease activity in both realms—and we found such relationships, again extending knowledge and supporting data in this realm.

Our study had limitations. First, our study was cross-sectional, and longitudinal studies would be desirable to examine changes over time. Second, although our study was larger than some studies, it was still relatively small and was all female; a mixed gender grouping is always desirable. Third, although we used validated markers of telomere shortening, others used telomere fragment lengths, a more direct measure.

In conclusion, our study found telomere shortening and significant oxidative stress in our RA patients compared to controls. Further, we found a relationship between telomere shortening and RA disease activity.

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Compliance with ethical standards

Disclosures None.

Ethics approval The Ethics Committee of Assiut University Hospitals approved study protocols, and each participant signed a written informed consent.

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