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Oxidative stress biomarkers as indicator of chronic inflammatory joint diseases stage

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ABSTRACT

Objective: To evaluate the participation of oxidative stress (OS) on chronic inflammatory joint disease (CIJD), as well as its possible use as a diagnostic biomarker.
Patients and methods: The study population comprised 29 patients with CIJD: 18 with rheumatoid arthritis (RA: 13 active/5 inactive); 11 with ankylosing spondylitis (AS: 7 active/4 inactive) and 13 healthy subjects. Activity of the disease was assessed by: RA patients, Disease Activity Score (DAS 28) and AS patients by means of Bath Ankylosing Spondylitis Disease Activity Index (BASDAI). Oxidative stress biomarkers were determined in plasma using spectrophotometrical techniques. The statistical analysis was carried out using the SSPS statistical package.
Results: Active CIJD showed a high oxidative stress characterized by increases in oxidative damage markers and a reduction in antioxidative systems, together with a higher myeloperoxidase (MPO) concentration. Inactive CIJD only showed changes in oxidized glutathione (GSSG) and reduced glutathione (GSH)/GSSG ratio levels, without changes in oxidative damage parameters or in antioxidative systems.
Conclusions: Our data revealed that: i) CIJD presents with a high oxidative stress; ii) inactive CIJD shows a production of reactive species without triggering oxidative damage and maintaining red-ox homeostasis, and iii) the combination of oxidative stress biomarkers may be used as markers of active-inactive stages of CIJD.

Biomarcadores de estrés oxidativo como indicadores de actividad en la enfermedad articular inflamatoria crónica

Palabras clave:
Artritis reumatoide
Daño oxidativo
Enfermedad articular inflamatoria crónica
Espondilitis anquilosante
Especies reactivas del oxígeno

RESUMEN

Objetivo: Evaluar la participación del estrés oxidativo (EO) en la enfermedad articular inflamatoria crónica (EAIC), así como su posible uso como biomarcador diagnóstico.
Pacientes y métodos: Se estudiaron 29 pacientes con EAIC: 18 con artritis reumatoide (AR): 13 activos/5 inactivos; y 11 con espondilitis anquilosante (EA): 7 activos/4 inactivos, y como grupo control, 13 sujetos sanos. Los pacientes fueron clasificados según los siguientes criterios de actividad: escala de actividad de la enfermedad (DAS-28) para AR, e índice de actividad de enfermedad (BASDAI) y la escala visual analógica (EVA) de dolor nocturno para EA. Las concentraciones plasmáticas de los biomarcadores de estrés oxidativo fueron cuantificadas mediante técnicas espectrofotométricas y el análisis estadístico realizado, mediante el programa estadístico SPSS.
Resultados: Los pacientes con EAIC activa presentan un intenso EO, caracterizado por elevación de los parámetros indicadores de daño oxidativo y disminución de los sistemas antioxidantes, junto con una mayor cantidad de mieloperoxidasa. En los pacientes con EAIC inactiva sólo encontramos cambios en los niveles de glutatión oxidado (GSSG) y en el cociente glutatión reducido (GSH)/GSSG, y no en los indicadores de daño oxidativo ni en los sistemas antioxidantes.
Conclusiones: Nuestros datos indican que: a) los pacientes con EAIC activa presentan un intenso EO; b) la EAIC inactiva cursa con producción de especies reactivas sin llegar a desencadenar daño oxidativo y manteniendo la homeostasis reducción-oxidación, y c) los biomarcadores de EO podrían ser utilizados como indicadores del estado de actividad-inactividad de la EAIC.

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Introduction

Oxidative stress (OS) is defined as the imbalance between oxidants and antioxidants in the body in favor of the former. The production of reactive oxygen species (ROS) (pro-oxidants and oxidants) physiologically occurs in all living organisms, and its main endogenous source is the mitochondrial electron transport chain.[1]

The oxidative damage that characterizes OS has been associated with different neurodegenerative and connective tissue diseases, ischemia-reperfusion processes and inflammation, among others.[2] Recent studies by different groups indicate that this biochemical phenomenon can be found as the pathophysiological basis of chronic inflammatory joint disease (CIJD), conditioning its evolving status.[3,4]

Based on this background, this study suggests that OS is associated with the activity status of patients with CIJD, so that changes in some biomarkers of OS might be associated with disease status (active or inactive) and therefore could be used as an early and effective marker of this condition in patients with this type of pathological processes. To assess our hypotheses and achieve the stated objective, we have studied a total of 42 subjects (13 healthy controls and 29 patients with CIJD, 20 active and nine inactive) quantified the levels of lipid peroxidation products (LPO), protein carbonyl (PC) , total glutathione (GT), glutathione (GSH), oxidized glutathione (GSSG) and GSH / GSSG ratio, along with the concentration of myeloperoxidase (MPO) (enzyme classification number [EC] 1.11.1.7), and the enzymatic activities of superoxide dismutase (SOD) (EC: 1.15.1.1), glutathione peroxidase (GPx) (EC: 1.11.1.12) and catalase (EC: 1.11.6).

Material and methods

Patients and controls

This was a cross-sectional study that included a total of 29 patients with CIJD (20 active and nine inactive) treated at the Rheumatology Service, Hospital Universitario Reina Sofia in Cordoba and who gave informed consent. Eighteen patients met the criteria of the American Rheumatism Association (ARA) for rheumatoid arthritis (RA) (13 active and 5 inactive) and 11 the New York modified criteria for ankylosing spondylitis (AS) (7 active and 4 inactive). Thirteen healthy subjects were studied as controls. The patients were classified as active or inactive according to: 1) a value of > 3.2 in the scale of disease activity (DAS-28)[5] calculated with the erythrocyte sedimentation rate (ESR), 2) a score > 4 on the Bath AS disease activity index (BASDAI),[6] 3) > 4 on a visual analog scale (VAS) of pain at night (0-10) for AS.

The study exclusion criteria were alcohol consumption, smoking, use of vitamins, infection and / or treatment with drugs with known antioxidant effects. All patients were told to stop taking NSAIDs 48 h before sampling, while folic acid was suspended in RA patients a week earlier.

The study of biomarkers of OS was carried out in plasma. Blood samples were taken from median ulnar or basilic veins and placed in tubes containing EDTA as an anticoagulant. They were then centrifuged at 2.000 rpm for 10 min, excess (plasma) was collected and frozen at –80 ºC until analysis.

Biomarkers of oxidative stress

The following parameters were measured as indicators of oxidative status: The amount of MPO, levels of LPO products (malondialdehyde and 4-hidroxyalkenals), GT and GSH concentration through commercial assays provided by Oxis International (Portland, OR, USA: MPO, LPO and GSH-586 GSH-420 and 400, respectively).

PCs were quantified by the method proposed by Levine et al,[7] based on the reaction with 2,4 dinitrophenylhydrazone in acid. GPx activity was assessed by the technique of Floh and Gunzler,[8] based on the oxidation of reduced nicotinamide adenine dinucleotide phosphate (NADPH) to oxidized nicotinamide adenine dinucleotide phosphate (NAD^+), catalyzed by a limiting concentration of glutathione reductase. For its part, catalase activity was obtained by the range of decomposition of H_2O_2, following the procedure of Aebi,[9] while SOD activity was assessed by the method of Sun et al[10] involving the inhibition of the nitrotetrazolium blue reduction of the xanthine oxidase system for SOD. On the other hand, GSSG levels were calculated using the following equation: GT=GSSG–GSH.

Statistical analysis

The statistical analysis was performed using SPSS® 11.0 (SPSS Ibérica, Madrid, Spain) for Windows®. We performed the Shapiro-Wilk test for the assessment of normal distribution. We performed an ANOVA and Bonferroni test as *post hoc* contrasts to compare the mean values of the OS parameters between different groups of patients according to disease activity. All contrasts were bilateral and values were considered significant if $P<.05$.

Results

Twenty-nine patients (nine active and 20 inactive) were compared with 13 healthy subjects (control). At the time of the evaluation of patients with active AS showed a mean BASDAI of 4.9±1.2, whereas the inactive presented values of 1.7±0.7. For its part, the mean DAS-28 for patients with active RA was 3.9±0.9, while the inactive showed values of 1.5±0.4. Compared with the control group, patients with active CIJD presented higher levels of ESR and CRP, while patients with inactive CIJD did not change significantly in comparison to healthy subjects (Table 1). The rest of the demographic variables analyzed are listed in Table 1.

Compared with controls, the results show that patients with active CIJD present great OS characterized by high levels in LPO, PC, GSSG and MPO activity and decreases levels of GSH, the GSH/GSSG ratio and the activity of antioxidant enzymes (catalase, SOD and GPx), while no changes were found in the levels of GT (Table 2).

For their part, patients with inactive disease only showed significant changes in levels of GSSG and the GSH/GSSG ratio compared to controls (Table 2). As shown, lower values of LPO, PC and MPO activity with respect to active CIJD and higher values in GSH, GSH/GSSG ratio, GPx, catalase and SOD (Table 2).

Table 3 shows the correlation analysis between the variables studied in this work. This table includes only statistically significant associations found in the total CIJD (AR and AS) and their subgroups (active and inactive). Positive association present in the total number of patients analyzed: 1) LPO with WBC and ESR in all patients, 2) GSSG with DAS-28 and EVA, and 3) with MPO LPO, PC, GSSG, WBC and ESR, while the correlation was negative in: 1) GSH with DAS-28 and VSG, 2) GSH/GSSG ratio with DAS-28 and VAS, and 3) MPO with GSH, GSH/GSSG ratio, GPx, catalase and SOD. For its part, in the correlation study stratified by subgroups, we only obtained statistically significant associations in the subgroup of inactive CIJD characterized by positive correlations between LPO and MPO-VAS-VAS, and an inverse correlation between GSH-ESR.

Discussion

The results of our study show that active CIJD causes an intense OS characterized by: 1) an increase in biomarkers of oxidative damage, and 2) a decrease in peripheral antioxidants. While patients with inactive CIJD, although showing significant changes in MPO values, GSSG and GSH/GSSG ratio, have no alterations in oxidative biomarkers (LPO and PC), nor in the studied antioxidant system (GSH, SOD, catalase

Table 1
Demographic characteristics of healthy subjects (controls) and patients with chronic inflammatory joint disease

	Controls	Total patients	Inactive	Actives
Number	13	29	9	20
Gender, male/female	3/10	13/16	5/4	8/12*,•
Age, years	37.09±11.70 (22-53)	48.32±12.70 (27-73)	69.50±4.95 (27-73)	52.67±11.85 (33-64)
ESR, mm/h	10.82±5.78	22.33±15.79	10.50±4.95	29.67±8.96**,••
CRP, mg/l	4.01±7.19	14.63±18.71	3.20±0.71	17.07±11.64*,•
Leukocytes, $\times 10^3$/ml	6.00±4.34	7.48±1.86	6.95±2.05	9.07±2.03*,•

Values represent mean ± SD.
*P<.05 vs control group.**P<.001 vs control group. •P<.05 vs inactive group. ••P<.001 vs inactive group.
CRP indicantes C reactive protein; ESR, erythrocyte sedimentation rate.

Table 2
Biomarkers of oxidative stress

	Controls	Patients	
		Inactive	Active
MPO, ng/ml	68.8±4.8	82.5±5.1*	113.5±9.2*,••
LPO, nmol/dl	65.1±20.1	31.1±13.7	215.6±87.0*,••
PC, nmol/dl	9.6±1.2	12.5±1.9	20.5±5.0*,••
GT, nmol/l	51.9±10.2	58.8±8.3	57.8±56.2
GSH, nmol/dl	43.2±8.4	36.1±3.5	21.1±6.3*,••
GSSG, nmol/l	8.9±6.5	22.6±6.5*	36.7±7.6*,••
GSH/GSSG ratio	8.8±12.8	1.71±0.5*	0.6±0.3*,•
GPx, U/dl	12.5±1.8	10.4±1.6	5.8±1.3*,••
Catalase, U/dlx100	11.8±1.8	10.8±1.3	5.7±1.2*,••
SOD, U/dl	52.2±5.6	47.2±12.2	27.2±6.5*,••

*P< .001 vs control group. •P<.01 vs inactive group. ••P<.001 vs inactive group. GPx indicates glutathion peroxidase; GSH, reduced glutathion; GSSG, glutathione oxide; GT, total glutathion; LPO, lipoperoxidation; MPO, myeloperoxidase; PC, carbonilated proteins; SOD, superoxide dismutase.

Table 3
Correlation between the quantified parameters in patients with chronic inflammatory joint disease

	Total (29)	
Parameters	r	P
LPO-leukocytes	0.559	.008
LPO-VSG	0.937	.019
LPO-VAS		
GSH-VSG	–0.603	.013
GSH-DAS-28	–0.681	.043
GSSG-VAS	0.499	.042
GSSG-DAS-28	0.670	.048
(GSH/GSSG ratio)-VAS	–0.479	.050
(GSH/GSSG ratio)-DAS-28	–0.658	.050
MPO-LPO	0.574	.002
MPO-PC	0.597	.001
MPO-GSH	–0.730	.000
MPO-GSSG	0.687	.000
MPO-(GSH/GSSG ratio)	–0.748	.000
MPO-GPx	–0.842	.000
MPO-CAT	–0.865	.000
MPO-SOD	–0.718	.000
MPO-leukocytes	0.464	.034
MPO-VSG	0.530	.024

CAT indicates catalase; DAS-28, disease activity score;GPx, glutathione peroxidase; GSH, reduced glutathione; GSSG, glutathione oxide; LPO, lipoperoxidation; MPO, myeloperoxidase; PC, carbonilated proteins; SOD, superoxide dismutase; VAS, visual analog scale.

and GPx). The latter situation indicates two possible phenomena: 1) an incipient increase in the production of ROS, which can be inferred from the increase in the oxidation of molecules of GSH and evidenced by increases in GSSG levels and the reduction of the GSH/GSSG ratio, and 2) the effectiveness of antioxidant systems in redox homeostasis, reflected in the lack of changes in direct indicators of oxidative damage (LPO and PC) despite the increase in oxidized molecules.

CIJD is an autoimmune condition characterized by inflammatory events leading to joint destruction. Probably the most common and studied is RA, which affects approximately 1% of the adult population in western countries.[3] Currently, knowledge of the molecular and cellular mechanisms involved in the initiation and development of these disease processes is incomplete. Different researchers have been involved in determining the origin, onset and evolution of ROS.

ROS seem to have a relevant role in the development of alterations in these processes.[3,11] Data from this study corroborate previous data from our group and other groups,[3,4,11-14] showing the existence of a highly significant increase in the levels of lipid and protein oxidation in patients with active CIJD while inactive patients to have similar levels to those of healthy subjects (controls). Nonetheless, patients with inactive CIJD show significant changes in GSSG and the GSH/GSSG ratio when compared with controls and active patients. Results indicate that an intermediary participates in the production of ROS and oxidation of molecules in inactive patients acting as a mediator between their health status and active disease.

On the other hand, there is no consensus in the scientific literature on fluctuations in the activity of antioxidant enzymes, reporting both increases and decreases.[3,12] In this sense, our data clearly show a decrease in the activity of the studied antioxidant enzymes (SOD, catalase and GPx) in patients with active disease, a situation that could be explained by a saturation of the enzymatic antioxidant systems, as well as inhibition of antioxidant enzymes, as suggested by Jira et al, having the potential to inhibit SOD through hydrogen peroxide.[15] Our results also reveal a decrease in the relationship between antioxidant activity (SOD/GPx+catalase) in both active and inactive patients. This situation increases with oxidation biomarkers indicating, according to the constructs by Sánchez-Rodríguez et al,[16] the existence of a deficiency in the extracellular antioxidant system.

Along with the decrease in antioxidant enzyme systems, we see increases in MPO enzyme activity parallel to increased levels of circulating peripheral neutrophils, both in patients with active and inactive disease. MPO is used by macrophages to produce ROS with bactericidal action. According to different authors, this would represent the link between the increased inflammatory response and OS in active CIJD.[12,14] These observations are backed directly by studies showing how different cytokines (TNF-α, IL-6, IL-8 and INF-γ) cause increased levels of vascular adhesion molecules (which attract leukocytes to joints) and activate proinflammatory genes through the NF-κB pathway, a mechanism which involves ROS,[10] and indirectly as in those studies in which citokine inhibitors such as infliximab (anti-TNF-α) induce a reduced inflammatory response, accompanied by a higher redox status and imoproved patient condition.[4,10] Additionally, and without being the aim of this study, this data indirectly support the role of ROS in the activation of proinflammatory signals, and its effects on endothelial dysfunction and atherogenesis in situations of dyslipidemia in patients with RA.[17,18]

Finally, we think that some of the most relevant data found in this study are the correlations between MPO activity, biomarkers of OS, nonspecific markers of inflammation and leukocytes in all of the

studied patients. Evidence linking the inflammatory response, ROS production and oxidative damage with disease status (active-inactive) demonstrate the dominant role played by OS in the progression of CIJD.

In summary, our results show the involvement of MPO and OS in CIJD, and the possibility of using different OS biomarkers as indicators of activity-inactivity in CIJD.

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