

# Impaired glutathione reductase activity and levels of collagenase and elastase in synovial fluid in rheumatoid arthritis

L. Bazzichi<sup>1</sup>, M.L. Ciompi<sup>1</sup>, L. Betti<sup>2</sup>, A. Rossi<sup>2</sup>, D. Melchiorre<sup>1</sup>, M. Fiorini<sup>2</sup>,  
G. Giannaccini<sup>2</sup>, A. Lucacchini<sup>2</sup>

<sup>1</sup>*Rheumatic Diseases Unit, Medica Santa Chiara Hospital, University of Pisa;*

<sup>2</sup>*Department of Psychiatry, Neurobiology, Pharmacology and Biotechnology,  
University of Pisa, Pisa, Italy.*

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## Abstract

### Objective

*To test the activity of elastase, collagenase and glutathione reductase in the synovial fluid (SF) of patients with rheumatoid arthritis (RA) and in patients with osteoarthritis (OA); to correlate the elastase and collagenase activity with the glutathione reductase activity, which is important for the inactivation of oxygen free radicals.*

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### Methods

*24 patients affected by osteoarthritis and 24 patients affected by rheumatoid arthritis took part in the study. We measured elastase activity towards the substrate metoxysuccinyl-alanyl-alanyl-prolyl-valyl-p-nitroanilide (MeOSuc-ala-ala-pro-val-p-NA) which is highly specific for elastase, and insensitive to the other serine proteases, such as cathepsin G; collagenase activity was measured using [<sup>14</sup>C]-acetylated collagen as the substrate. Glutathione reductase activity was measured following the oxidation of nicotinamide adenine dinucleotide phosphate reduced (NADPH) in the presence of oxidized glutathione (GSSG).*

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### Results

*The concentrations of elastase, collagenase and glutathione reductase were statistically higher in patients with RA than in patients with OA. Moreover, in the SF of patients with RA we found positive correlation between enzyme activity levels.*

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### Conclusion

*These results confirm a high activity of collagenase and elastase in the SF of patients with RA, which is about 30 times higher than that found in the SF of patients with OA. These data underline the synergic action of these enzymes in the pathogenesis of joint damage. RA patients also exhibit higher levels of glutathione reductase, which is important for the detoxification pathway of oxygen free radicals. However, compared with findings for collagenase and elastase, the increase in glutathione reductase is only three times higher than level found in the SF of OA patients. The limited increase in glutathione reductase activity during the inflammatory process might lead to an insufficient protective effect at the joint level in rheumatoid arthritis.*

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### Key words

Collagenase, elastase, glutathione reductase, rheumatoid arthritis.

Laura Bazzichi, MD, Specialist in Rheumatology; Maria Laura Ciompi, MD, Associate Professor; Laura Betti, PhD; Alessandra Rossi, PhD; Daniela Melchiorre, MD, PhD; Marcello Fiorini MD, PhD; Gino Giannaccini, PhD, Researcher; Antonio Lucacchini, PhD, Full Professor.

Please address correspondence and reprint requests to: Prof. Antonio Lucacchini, Dipartimento di Psichiatria, Neurobiologia, Farmacologia e Biotecnologie, University of Pisa, Via Bonanno 6, 56126, Pisa, Italy. E-mail: lucas@farm.unip

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## Introduction

The destruction of joint cartilage that takes place during the inflammatory process is mainly caused by the action of proteases such as elastase and collagenase, which are potent enzymes secreted by inflammatory cells. In determination of joint damage, the synergic action of the reactive oxygen species (ROS) produced during activation of the neutrophil is also important. Elastase is a proteolytic enzyme which is deposited inside neutrophils; it is released in certain conditions, such as inflammation, and its action determines the degradation of collagen and elastin, which, together with glycosaminoglycans (GAGs), are the components of the extracellular matrix of joint cartilage; in normal conditions, it is inactive, because it is bound to inhibitors such as the alpha-1 proteinase inhibitor, or alpha-2 macroglobulin. The enzyme collagenase was the first member of the metalloproteinases (MMPs) family that was specifically identified. This family also includes stromelysin and gelatinase, which are secreted in a proenzyme form which, once activated, can remove connective tissue and joint damage (1).

Collagenase cleaves the triple helix of collagen at a specific point, but it may attack all the tissue matrix components. All active MMPs collagenases are inhibited by tissue inhibitors of metalloproteinases (TIMPs) and alpha-2-macroglobulin, which act as a control mechanism of these potent enzymes.

In cases of osteoarthritis, where the inflammatory phenomena are less intense, the elastase and collagenase produced in limited quantities by chondrocytes are secreted and neutralised by the production of inhibitors, and assume a lesser significance in the pathogenesis of joint damage. In cases of arthritis, on the contrary, elastases and collagenases are not only produced by chondrocytes, but are also released in large quantities by inflammatory cells, such as polymorphonucleates and synovialocytes (2); they are thus extremely important in determining joint damage. Together with elastases and collagenases, an important role in joint destruction is played by ROS. The production

of oxygen free radicals takes place by means of the activation of the neutrophil membranes by various substances, such as immune complexes, crystals, cytokines C5a, Fc fragments of immunoglobulins, etc., all of which are substances present during the inflammatory process. High levels of products of free radical reactions have been reported in the blood sera and synovial fluids of patients with rheumatoid arthritis (3). Collagen can be altered directly or indirectly by oxygen radicals: hydroxyl radicals have a direct effect, cleaving collagen, in the presence of oxygen into small peptides; furthermore, free radicals can begin the cleavage of collagen, making it more sensitive to proteolytic enzymes (4). Even more important may be the indirect effects of radicals, via the activation of latent collagenase and neutralization of protease inhibitor activity (5, 6). Neutrophils release and activate the latent enzymes, gelatinase and collagenase; the ability of neutrophils to activate these enzymes depends on myeloperoxidase, HOCl and H<sub>2</sub>O<sub>2</sub>.

The inactivation of oxygen radicals takes place by the glutathione system, reduced and oxidised by glutathione reductase, an important enzyme for the regeneration of oxidised glutathione to its reduced form. The H<sub>2</sub>O<sub>2</sub> that is formed can be metabolised to H<sub>2</sub>O, with the concomitant oxidation of reduced glutathione (GSH). This reaction is considered to be a detoxification pathway under most conditions (7).

Various papers have reported high levels of MMPs in the synovial fluid of arthritis patients, correlating them directly with joint damage, considering also the levels of TIMPs and the production of free radicals, which are found to be high (8).

The aim of this study was to evaluate the activity of collagenase, elastase and for the first time the activity of glutathione reductase in the SF of patients with osteoarthritis or rheumatoid arthritis, and to study the correlation existing between these enzymes.

## Patients and methods

### Materials

Elastase, neutrophil elastase substrate,

MeOSuc-ala-ala-pro-val-p-NA, glutathione reductase, NADPH, collagen type III from calf skin, collagenase type IV from *Clostridium histolyticum* 480 U/mg for the standard curve, trypsin from bovine pancreas 10,000 BAEE U/mg and trypsin inhibitor type II soybean, were from Sigma-Aldrich. [ $^{14}$ C]-acetylated collagen (400,000 dpm) was obtained from [ $^{14}$ C]acetic anhydride (specific activity 10 mCi/mmol) from NEN (Boston, MA, USA). All other reagents were obtained from normal commercial sources.

### Subjects

24 patients affected by osteoarthritis (10 male and 14 female), aged 47-72 years, and 24 patients affected by rheumatoid arthritis (9 male and 15 female) aged 27-63 years took part in the study. OA was established on the basis of the ACR Clinical Classification Criteria for osteoarthritis of the knee. All OA patients hadn't a history of acute or chronic inflammatory disease, and none of them had any inflammatory condition during the last month. RA subjects had a minimum duration of disease of 12 months. Nine of the 24 patients were seronegative. All RA patients were in Steinbrocker functional class II and III and with active disease that was defined by the presence of active arthritis (with presence of swelling and/or limitation of movement or tenderness) at least in one joint at the clinical examination, and elevated C-reactive protein (CRP) level and erythrocyte sedimentation rate (ESR). RA diagnosis was clinically established as definite or clinical on the basis of American Rheumatism Association (ARA) criteria (9).

All patients included in the study were treated with non-steroidal anti-inflammatory drugs and with low doses of steroids. Written consent was obtained from all the subjects, after a full explanation of the procedure. All the procedures followed were in accordance with the standards of the responsible local committee.

### Synovial fluid studies

Synovial fluids (SF) were aspirated from knee joints of patients with OA

and RA, drug-free at the time of sampling. Immediately after aspiration, the SFs were centrifuged at 1700 g for 15 minutes at 4°C. The supernatants were collected and all the samples were kept at -20°C until the moment of use. Synovial fluids were previously treated with hyaluronidase.

### Assays

*a) Elastase.* SFs were assayed for elastase activity by spectrophotometric assay using MeOSuc-Ala-Ala-Pro-Val-p-NA as the substrate, which is highly specific, but does not assay the other serine proteases (such as cathepsin G) (10). The basis for this assay is the release of 4-nitroanilide by the esterase action of elastase, which is followed by measurement of the increase in absorbance at 410 nm. In brief, the substrate was used at a final concentration of 4 mM in 100 mM Hepes buffer (pH 7.5) containing 500 mM NaCl and 2% DMSO. A volume of 50  $\mu$ l SF samples was added to enzyme substrate in 2 ml reaction volume, mixed thoroughly and measured. Incubation was at 37°C and elastase activity was quoted as UE/l (one unit releases 1  $\mu$ M p-NA/minute at 37°C).

*b) Collagenase.* Synovial fluids were activated with trypsin and then treated with trypsin inhibitor. The collagenase assay was performed using [ $^{14}$ C]acetylated collagen as the substrate (11). The [ $^{14}$ C]acetylated collagen solution (60  $\mu$ g) was added to the lower side of each well in such a way that only half the surface was covered. The plates, left at the 45° angle, were incubated at 37°C for 1 hour as described by Johnson-Wint, to polymerize the collagen to a fibrillar gel, and were then carefully immersed in distilled water (2 liters for 4 plates) at room temperature and left for 1 hour to remove the phosphate buffer from the gels (12). Water was shaken gently out of the wells so as to retain all of the collagen, and the plates were left standing on end in air at room temperature (22°C) overnight to allow the gels to dry to collagen films.

In order to assay enzyme-containing SF for collagenase activity, 50  $\mu$ l of straight SF were added per well, and the assay plate was incubated at 37°C

for 0.5 to 3 hours. The entire supernatant containing radioactive collagen degradation products was then collected with a micropipet from the non-collagen coated side of each well, so as not to disturb the undegraded film, and was counted in a scintillation spectrometer. Each assay contained a buffer control in which wells were incubated with the buffer, and also a trypsin (0.01%) control, to measure the amount of denatured collagen in the substrate. Experimental values for enzyme SF were obtained by subtracting the buffer control counts per minute from measured counts per minute. Total counts per minute per well were determined by complete digestion of four wells with an excess of crude bacterial collagenase (4 mg/ml in buffer).

*c) Glutathione reductase (GR).* GR catalyzed the reaction following way: GSSG (oxidised glutathione) + NADPH + H<sup>+</sup> 2GSH (reduced glutathione) + NADP<sup>+</sup>

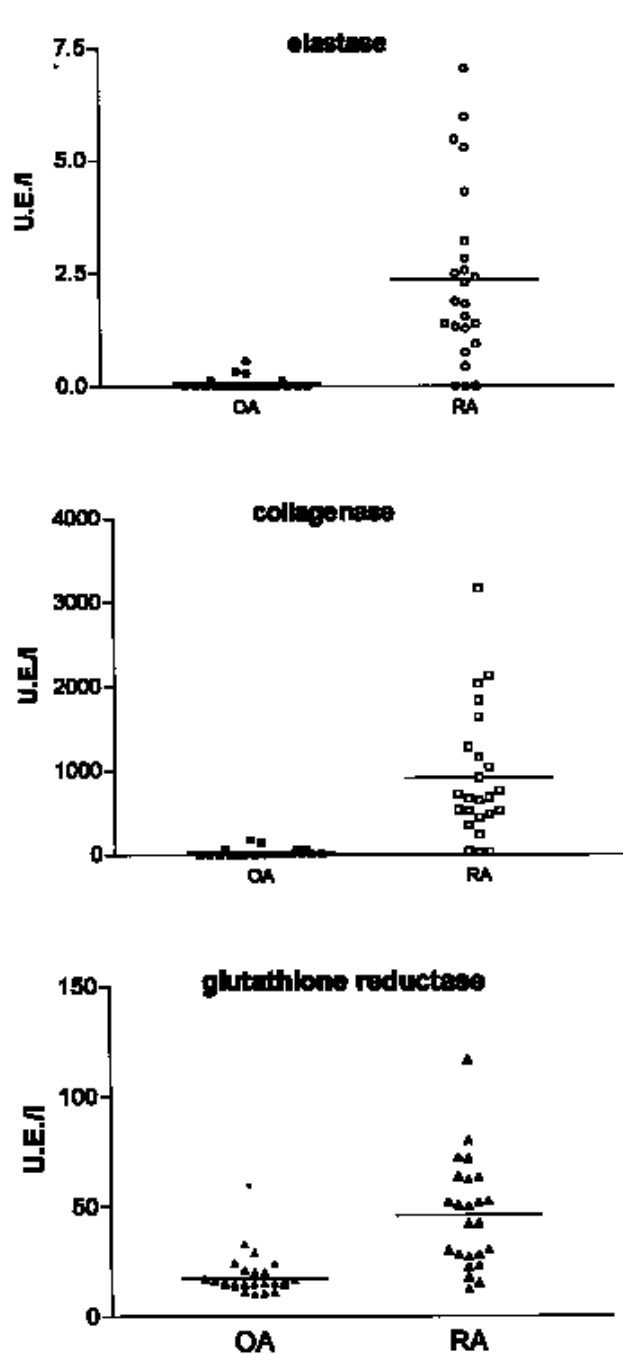
In this way, we quantified, following the reaction, the oxidised GSSG in SFs at 340 nm by measurement of variation in absorbance, which is a function of the oxidation of NADPH (13). The reaction mixture contained 50 mM Tris-HCl buffer (pH 7.6), 0.1 mM EDTA, 0.14 mM NADPH, 1 mM GSSG. The enzyme source was 50  $\mu$ l SF in 2 ml reaction volume. Incubation was at 37°C, and the specific enzyme activity was expressed as UE/l (one unit releases 1  $\mu$ M of GSSG/minute at 37°C).

### Statistical analysis

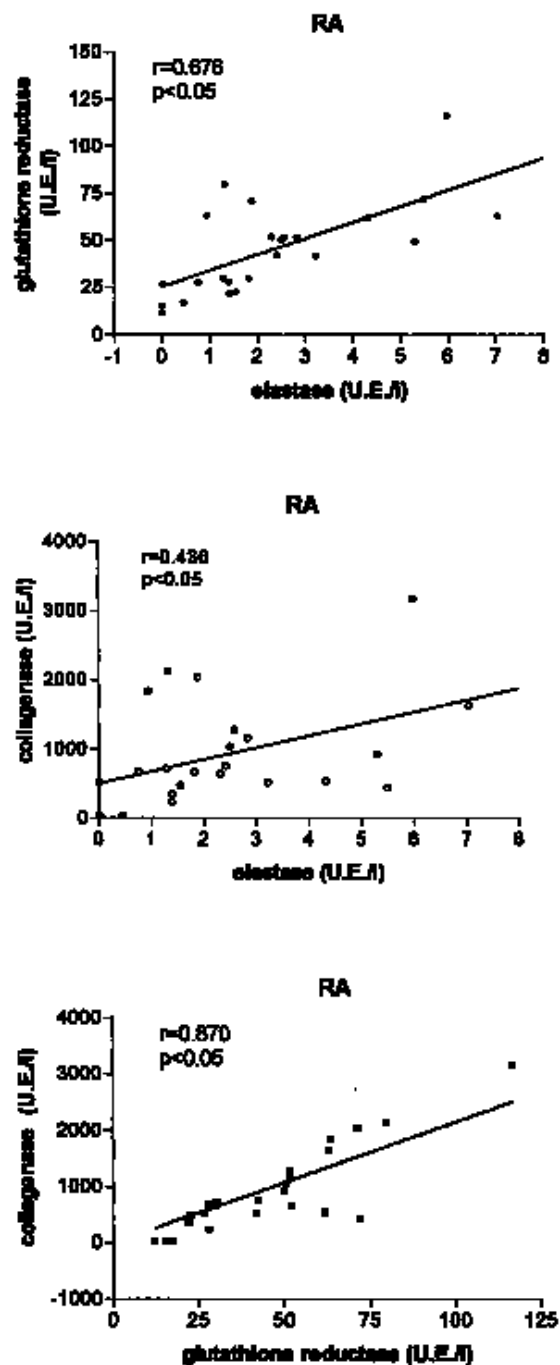
Statistical analysis was performed using multi-group variance analysis (ANOVA) according to Scheffe's "post-hoc" procedure. Significant p results were assigned to values lower than 0.05. Correlation was evaluated by Spearman correlation test.

### Results

The individual values for the activity of elastase, collagenase and glutathione reductase, and their means in the SF of patients with RA and patients with OA are shown in Figure 1 and Table I. A very low activity of the enzymes measured was found in the patients with



**Fig. 1.** Distribution of the individual values for elastase, collagenase and glutathione reductase in SF of patients with osteoarthritis (OA) and patients with rheumatoid arthritis (RA). The means (horizontal line) are depicted as well.



**Fig. 2.** Direct relationship between glutathione reductase (U.E./l) and elastase (U.E./l), between collagenase (U.E./l) and elastase (U.E./l) and between collagenase (U.E./l) and glutathione reductase (U.E./l) activity in SF from RA (n = 24).

OA; in particular, it was only just possible to measure the values for elastase in OA patients. The differences between the mean enzyme activity in the SF of patients with RA and patients with OA were significant. The mean values for all these enzyme assays were significantly higher in SF from RA than from OA patients. In particular, the mean elastase activity was higher in patients

with RA (2.37 UE/l) than in patients with OA (0.07 UE/l), statistical analysis revealed a significant difference between RA and OA ( $p < 0.05$ ). The mean glutathione reductase activity was higher in patients with RA (45.88 UE/l) than in patients with OA (17.17 UE/l); statistical analysis showed a significant difference between RA and OA ( $p < 0.05$ ). The mean collagenase activ-

ity was higher in patients with RA (912 UE/l) than in patients with OA (30 UE/l), statistical analysis again showed a significant difference between RA and OA ( $p < 0.05$ ).

In the SF of patients with RA, we found a correlation between enzyme activities, a significant correlation was found between the synovial activity of glutathione reductase and elastase from

patients ( $r = 0.673$ ,  $p < 0.05$ ), between activity of collagenase and elastase from patients ( $r = 0.436$ ,  $p < 0.05$ ), and between collagenase and glutathione reductase ( $r = 0.870$ ,  $p < 0.05$ ) (Fig. 2). We didn't find any correlation between serum levels of CRP or SER and enzyme activity.

### Discussion

Elastases and collagenases are involved in the normal turnover of the connective tissue matrix, which takes place during growth and development; they are also involved during inflammation in joint destruction. It is not easy to measure the levels of these enzymes in synovial fluid, but our dosing system has proved to be highly sensitive for collagenase, and extremely specific for elastase, as a selective substrate is used for this enzyme which is not attacked by other serine proteases.

Glutathione is a tripeptide present in animal cells, where it performs a buffer action on sulfhydryl groups, with the

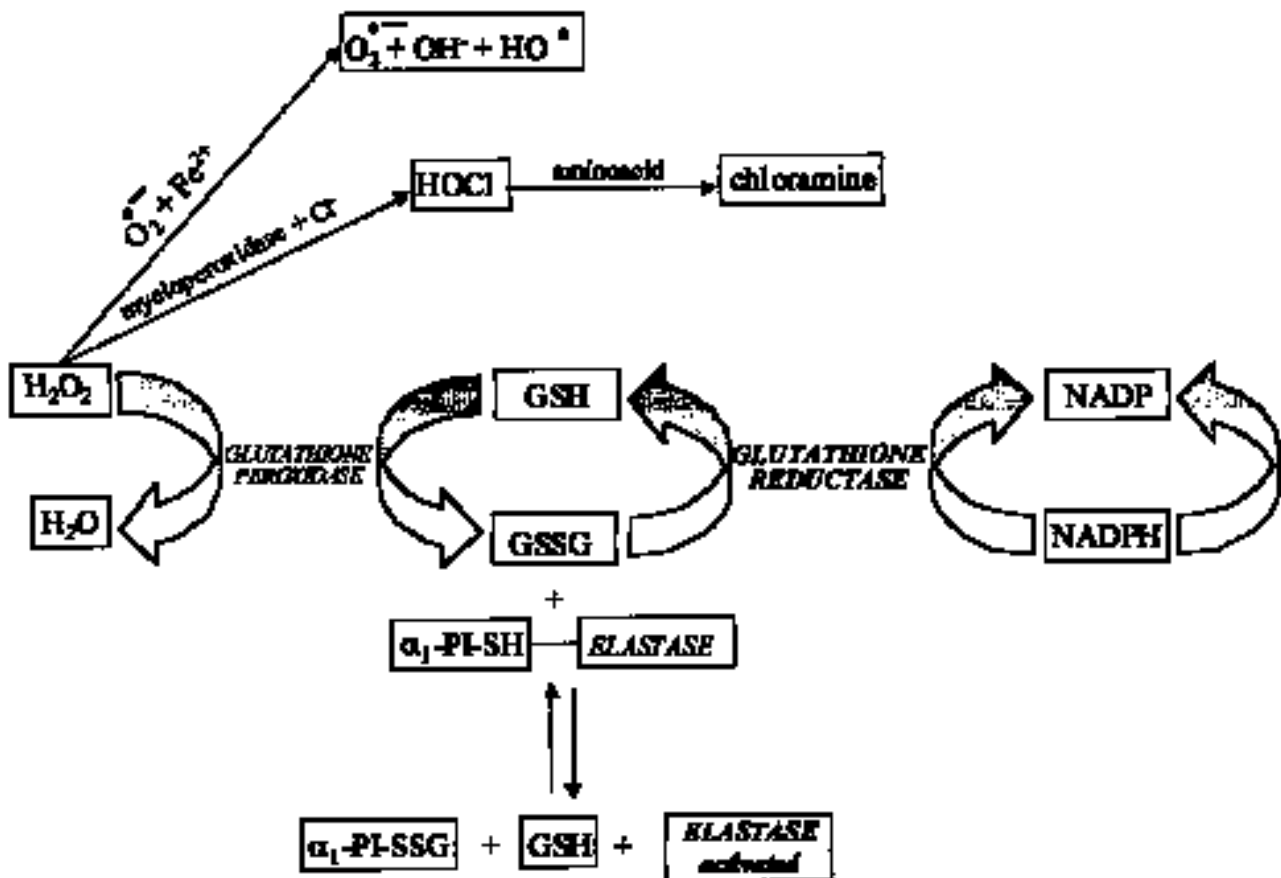
**Table I.** Mean value ( $\pm$  SEM) of elastase, collagenase and glutathione reductase activity in synovial fluids.

Disorder	Elastase activity (UE/l)	Collagenase activity (UE/l)	Glutathione reductase activity (UE/l)
Osteoarthritis	$0.070 \pm 0.028$ n = 24	$30 \pm 10$ n = 24	$17.17 \pm 1.16$ n = 24
Rheumatoid arthritis	$2.37 \pm 0.40^{**}$ n = 24	$912 \pm 157^{**}$ n = 24	$45.88 \pm 5.03^*$ n = 24

Statistically different from OA values (\* $p < 0.05$ ; \*\* $p < 0.001$ ). Multi-group variance analysis (ANOVA) according to Scheffe's "post-hoc" procedure.

aim of maintaining the protein cysteine residues in the reduced state, while it is oxidised. Reduced glutathione plays an important role in detoxification from  $H_2O_2$  and organic peroxides, which are substances produced in large quantities during the inflammatory process. High levels of glutathione reductase have been found in erythrocytes from patients with rheumatoid arthritis (14). We evaluated the activity of collagenases and elastases and glutathione reductase in the SF of patients with

osteoarthritis or rheumatoid arthritis, and found that in subjects with RA, the levels of the three enzymes studied are significantly higher compared with OA patients. Furthermore, in OA, the elastase levels are much lower, almost at the limits of detection. Even if we found a significant increase in the levels of all three enzymes in RA, it may be noted that while elastase and collagenase increase by about 30 times, glutathione reductase unexpectedly increases only three times, compared with



**Fig. 3.** Oxidoreduction reactions.

the values found in OA. This increase in glutathione reductase probably is inadequate to neutralise an excessive production of ROS, determining an increase in the oxidised form glutathione (GSSG). Thus the oxidised glutathione might react with the reduced form of the alpha-1 protease inhibitor, forming oxidised alpha-1 protease and reduced glutathione (GSH). The reduced form of glutathione should compensate for the damage caused by peroxides, by means of an oxidoreduction reaction, with the formation of H<sub>2</sub>O and GSSG (Fig. 3). On the other hand, the oxidised alpha-1 protease inhibitor has fewer possibilities of forming a complex with elastase, thus favouring the proteolytic action and an increase in joint damage (6).

Furthermore, for patients with RA, a positive correlation was found between the levels of elastase/glutathione reductase, elastase/collagenase, and glutathione reductase/collagenase, this might indicate a synergic action of the three enzymes, though the activity of glutathione reductase would appear to be insufficient to compensate for the proteolytic mechanism of ROS-induced protease. Furthermore, the increased production of GSSG might lead to the

inactivation of the elastase inhibitor, reducing the mechanism of feedback on this enzyme. This is an inflammatory process in which proteases are involved in a cascade mechanism of activation, which leads to joint damage that is difficult to control. It is desirable, on the basis of these data, to interrupt this activation at various levels, for example by neutralising the superoxide anions by means of selective mechanisms such as the potentiation of glutathione reductase, which appears to be a key enzyme in the mechanism of control over the inflammatory process at the synovial level.

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