

## Original Research Article

# Protein carbonyls and protein thiols in rheumatoid arthritis

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### ABSTRACT

**Background:** Oxidative stress (OS) has an important role in the pathogenesis and progression of rheumatoid arthritis (RA). OS causes protein modification, thereby impairing the biological functions of the protein. This study was conducted to assess the oxidatively modified protein as protein carbonyl content and the antioxidant status as protein thiols, and to study the association between protein carbonyls and protein thiols in RA.

**Methods:** Newly diagnosed RA patients who were not taking any disease modifying anti-rheumatic drugs were included into the study group (n=45) along with age and sex matched healthy controls (n=45). Serum protein carbonyl content and protein thiols were estimated.

**Results:** Elevated protein carbonyl content and decreased protein thiol levels (p<0.001) were observed in RA. A significant negative correlation was observed between protein carbonyl content and protein thiol levels (p<0.001).

**Conclusions:** Oxidative stress in RA is evidenced by enhanced protein oxidation and decreased antioxidant protein thiol levels. Decreased protein thiols may also reflect protein modifications leading to compromise in the antioxidant properties. This oxidant and antioxidant imbalance needs to be addressed by therapeutic interventions to prevent disease progression.

**Keywords:** Antioxidant, Oxidative stress, Protein carbonyl content, Protein thiols, Protein oxidation

### INTRODUCTION

Rheumatoid arthritis (RA) is an inflammatory autoimmune disease affecting diarthrodial joints. It was characterized by erosive synovitis, by infiltration of inflammatory cells into the synovium and synovial hyperplasia ultimately leading to the destruction of bone as well as articular cartilage and causing systemic complications including cardiovascular, pulmonary, psychological, and other skeletal disorders.<sup>1</sup> The prevalence of RA was around 1-2% of the world population with women being affected three times more often than men and more frequently seen in the 40 and 50 years age group.<sup>2</sup> The imbalance between oxidants and antioxidants due to increased chemical reaction or

insufficient antioxidant defense system results in oxidative stress (OS), which is also believed to play a pivotal role in pathogenesis and progression of inflammation in RA.<sup>3</sup>

Plasma proteins are targets of OS, which may enhance protein modifications such as protein carbonylation, generated directly by oxidation of amino acids and by alpha-amidation pathway or indirectly by lipid peroxidation products which form adducts with the protein.<sup>4</sup> Carbonylation is an irreversible and stable modification that is said to occur early on in the disease process and often leads to loss of protein function. Protein carbonyl groups remain in blood circulation for longer periods compared with other oxidative stress

markers such as glutathione disulfide (GSSG) or the lipid peroxidation product malondialdehyde. Plasma proteins have important physiological functions such as maintaining blood volume, transporting molecules, regulating endocrine systems and inflammatory responses.

Consequently, modifications to plasma proteins may exert diverse effects depending on the sites of damage. The frequency of protein modifications are reported to increase with age and chronicity of diseases. It is known that cells degrade oxidised proteins within hours and days, the mild oxidation of proteins as part of physiological process can be cleared by proteasome.<sup>5,6</sup> However, excessive oxidation and cross linking of proteins as occurs in oxidative stress conditions make them resistant to proteolytic degradation. Cells accumulate protein damage which reduce cell function and also may lead to cell death.<sup>7</sup> The abundant plasma proteins such as albumin and immunoglobulins are most commonly analyzed for damage. Modified proteins serve as important biomarkers. The presence of carbonyl groups in proteins has therefore been used as a marker of protein oxidation.<sup>8</sup> Protein thiols, which are the sulfhydryl (~SH) groups of amino acids such as cysteine, reflect the antioxidant status.

They act through a variety of mechanisms, such as components of the general thiol/disulfide redox buffer, as substrates for specific redox reactions (reduced glutathione), as metal chelators, as radical quenchers, and as specific reductants of individual protein disulfate bonds (thioredoxin).<sup>9</sup> Protein thiols can scavenge oxidants and thereby protect the cellular constituents from damage. Protein thiols also spare or conserve the other antioxidants. The present study was carried out in newly diagnosed RA patient who were naïve to treatment with the aim of evaluating protein carbonyl content, the stable and early marker of protein oxidative damage and the antioxidant status as protein thiols and to study the association between the protein carbonyl content and protein thiols.

## METHODS

The study was carried out in the department of Biochemistry of Sri Venkateswara Institute of Medical Sciences, Tirupati from October 2016 May 2017. The study subjects were selected from patients attending the Rheumatology clinic at Sri Venkateswara Institute of Medical Sciences, Tirupati and diagnosed with Rheumatoid arthritis as per the 1987 revised American Rheumatology Association criteria.<sup>10</sup> Individuals who had cardiovascular diseases (CVD), diabetes mellitus, renal failure, acute and chronic inflammatory disease, alcohol abuse, and those who were on anti-lipidemic and antioxidant drugs were excluded from the study. Newly diagnosed RA patients who were not taking any disease modifying anti-rheumatic drugs were included into the study group (n=45, females=36; 42.33±12.62yrs) along

with age and sex matched healthy controls (n=45, females=39; 40.15±11.59 yrs), after obtaining written informed consent from the subjects. The study was approved by the institutional ethics committee. After overnight fasting, venous blood samples were drawn into plain tubes, allowed to stand for 30 minutes and centrifuged for 15 min at 3000 rotations per minute, and aliquots of serum samples were stored at -80°C in deep freezer (Thermo Fischer Scientific, USA) until biochemical analysis.

**Biochemical Analysis:** Protein carbonyl groups were detected and quantified using 2,4-dinitrophenylhydrazine (DNPH), which reacts with protein carbonyls forming a Schiff base to produce the corresponding hydrazone, the absorbance of which was measured at 370nm.<sup>11</sup> Carbonyl levels were expressed as nanomoles of carbonyl per milligram of proteins. Protein thiols were estimated based on the reaction of thiols with Ellmen's Reagent, DTNB (5, 5'-dithiobis 2-nitrobenzoic acid) to give the mixed disulfide and 2-nitro-5-thiobenzoic acid (TNB) is a stable colour formed which is quantified by measuring the absorbance at 412nm.<sup>12</sup> The concentrations of thiol groups were calculated using a molar extinction coefficient of 13,600 M<sup>-1</sup> cm<sup>-1</sup>. Thiols were expressed as µmol/L.

Data distribution was studied using kolmogorov smirnov test. Data was normally distributed and values obtained were expressed as Mean ±Standard deviation. The difference in the mean between controls and cases was studied using parametric students 't' test. A 'p' value <0.05 was considered as statistically significant. Statistical analysis was performed using SPSS for windows version 11.5. Pearson's correlation analysis was performed between protein carbonyl content and protein thiols.

## RESULTS

Table 1 shows the difference in means between the control group and study group. A significant elevation of protein carbonyl content (p<0.001) and significant lower protein thiol (p<0.001) levels were observed in RA patients when compared to controls, indicating presence of oxidative stress and oxidant damage. The modification of protein structure due to oxidative stress is depicted by the increase in the protein carbonyl content and decrease in protein thiol levels. These protein structural alterations cause an accumulation of damage as protein carbonyls and a loss of protein antioxidant properties as protein thiols.

Table 2 shows the association between protein carbonyls and protein thiols in RA patients. A significant negative correlation (r = -0.406, p =<0.001) was observed between the parameters, indicating the decrease in antioxidant capacity is associated with oxidant damage. The decrease in protein thiol levels is indicative of decrease in antioxidant function due to oxidative stress which is

associated with oxidative stress induced protein modification presenting as protein carbonyl content.

**Table 1: Protein carbonyl content and protein thiols among study group and control group.**

Parameter (n=45)	Control group Mean ±SD	Study group Mean ±SD	p value
Protein carbonyl Content (nmol/mg of protein)	0.25±0.06	0.34±0.077	<0.001*
Protein thiols (µmol/L)	182.13±50.54	138±33.84	<0.001*

\*Statistically significant

**Table 2: Correlation analysis between protein carbonyls and protein thiols in RA.**

Parameter	R value	p value
Protein carbonyl Vs protein thiols	-0.406	<0.001*

\*Statistically significant

## DISCUSSION

Rheumatoid arthritis characterized by peripheral loss of cartilage due to up regulation of catabolic pathways, induced mainly by pro-inflammatory cytokines and reactive oxygen species (ROS).<sup>13</sup> Macrophages and polymorphonuclear cells present at the site of synovitis promote the formation of ROS and subsequent activation of inflammatory molecules.<sup>3</sup> OS contributes to joint inflammation and damage. Several factors contribute to the production of free radicals one of which is NADPH mechanism.<sup>14</sup> Oxidative stress is induced when the ROS generation exceeds the neutralizing capacity of the antioxidant system. ROS oxidizes IgG antibodies, induces rheumatoid factor production and oxidizes hyaluronic acid, there appears to be a relationship between protein oxidation, protein dysfunction, and it is known that oxidative modification of enzymes and structural proteins may play a significant role in the aetiology of diseases. Elevated levels of OS markers such as thioredoxin, sialic acid, and carbonyl content of proteins were reported in synovial fluid.<sup>15,16</sup>

We have also observed similar findings of elevated protein carbonyl content and decreased protein thiol levels in RA (Table 1). Plasma carbonyl status was assessed in juvenile chronic arthritis patients and a correlation was found between carbonyl groups and the activity of the disease.<sup>17</sup> The amount of protein carbonyl groups in plasma proteins reveals the intensity of free radical driven reaction which correlates with the extent of protein oxidation. Protein carbonyl groups may be introduced into proteins by reactions with aldehydes (4-hydroxy-2-nonenal, malondialdehyde) produced during lipid peroxidation or with reactive carbonyl derivatives (ketoamines, ketoaldehydes, deoxyosones) generated as a consequence of the reaction of reducing sugars or their

oxidation products with lysine residues of proteins (glycation and glycoxidation reactions). The presence of carbonyl groups in proteins has therefore been used as a marker of ROS-mediated protein oxidation, and several sensitive methods have been established for the detection and quantitation of protein carbonyl groups. It has been established that protein oxidation is associated with aging, oxidative stress, and a number of diseases. Studies have reported higher grades of protein carbonylation in plasma from RA patients compared with healthy controls.<sup>16-18</sup> Protein carbonyls are said to act as stable biomarkers of oxidative stress as they accurately indicate the level of the oxidative damage of proteins, they are an early indicator of the pathological process and they can be used to study the pharmacologic response to a therapeutic intervention.<sup>19</sup>

Elevated serum carbonyl protein is reported to be an important diagnostic tool in patients with acute myocardial infarction along with lipid profile and cardiac profile.<sup>20</sup> Protein carbonyl content were found to show significant negative correlation with the protein thiols (r = -0.406, p = <0.001) in RA patients in the present study (Table 2), indicating the association between OS and antioxidant status in RA. Protein thiols are also susceptible to oxidative modification by ROS which can be reversible such as intra and inter-protein disulfides, S-sulfenation, S-nitrosation, S-thiolation, S-sulfhydrylation, S-sulfenamiation and non-reversible hyper-oxidized (S-sulfination, S-sulfonation) redox states. Prolonged exposure to oxidants can result in irreversible modifications. These oxidative modifications are reported to be crucial to redox signaling pathways involved in cellular and physiological processes.<sup>21</sup> Reduced thiol status indicates unopposed and progressive OS. It was found that continuous exposure to even low levels of oxidants can eventually cause depletion of reduced glutathione.<sup>22</sup>

Upon treatment with an anti-inflammatory agent, infliximab, in RA, a short term decrease in protein carbonyl group levels were reported, and in the long-term treatment the levels were found to be similar to those of controls, suggesting the beneficial effects of infliximab on the protein oxidation process. Similarly, anti-tumor necrosis factor-α therapy induced a significant and sustained increase in thiol levels indicating improvement in antioxidant status, which correlated with a reduction in C-reactive protein concentrations. Early-stage detection of arthritic disease by robust biomarkers and effective lifestyle and/or pharmaceutical interventions could markedly decrease morbidity in RA and improve outcomes.

## CONCLUSION

Oxidative stress of rheumatoid arthritis enhances protein oxidation, reflected as protein carbonyl content. Decreased antioxidant protein thiols may also reflect protein modifications leading to compromise in the

antioxidant properties. The measurement of protein carbonyl content would be useful in assessing the disease severity. Therapeutic interventions to facilitate the antioxidant system along with carbonyl scavenging properties will prove beneficial in counteracting the damaging effects of OS on proteins.

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