

SERUM PROLIDASE ENZYME ACTIVITY AND OXIDATIVE STATUS IN PATIENTS WITH SCLERODERMA

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ABSTRACT

Objective: To assess serum prolidase enzyme activity and oxidative stress in patients with scleroderma (also known as systemic sclerosis, or SSc) and determine its relationship with serum oxidative status.

Methods: The study population consisted of SSc patients (n = 21) and healthy participants (n = 29). Serum prolidase enzyme activity, total antioxidant status (TAS), total oxidative status (TOS), oxidative stress index (OSI), and paraoxonase-1 (PON-1) levels were compared between the two groups.

Results: The mean duration of SSc involvement was 5 years; and the mean modified Rodnan skin score was 16. No statistically significant differences existed between the SSc groups and the control participants in terms of age, gender, TAS, PON-1, and haematocrit levels. Serum prolidase activity, OSI, and TOS levels were statistically significantly higher in SSc patients in comparison with controls participants. Also serum prolidase activity was statistically higher in Raynaud's phenomenon positive patients than Raynaud's phenomenon negative patients. But no correlation emerged between the serum prolidase activity and modified Rodnan Skin Score (P=0.235, r=0.304).

Conclusion: High prolidase activity may indicate critical biological activities relevant to pathological events in SSc, and this activity may be a biological indicator of disease. Further studies are needed to verify these findings.

Key words: bone marrow mesenchymal stem cells, mouse, culture.

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Introduction

Scleroderma (systemic sclerosis, or SSc) is a connective tissue disease involving fibrosis of the skin and internal organs and is characterized by increased deposition of extracellular matrix (ECM) proteins such as collagen and fibronectin⁽¹⁾. The main features of SSc include elevated levels of reactive oxygen species (ROS), vasculopathy, autoimmunity, and progressive fibrosis of the skin and other internal organs⁽¹⁾. SSc is commonly classified into two major clinical subsets, diffuse SSc and limited SSc. These common classification largely depend on the degree of skin involvement⁽²⁾. Activated fibroblasts play a key role in fibrosis in SSc⁽³⁾ but the etiology of SSc is still unknown⁽⁴⁾. It has been reported that oxidative stress contributes to clinical manifestations associated with SSc⁽⁵⁾. A pathogenetic correlation between SSc and oxidative stress has been reported in a study of Gabrielli et al⁽⁶⁾.

Prolidase, an enzyme found in human and animal tissues, plays an important role in collagen catabolism and synthesis. Prolidase activity is altered in fibrotic disease and chronic wounds and affected by oxidative stress^(7, 8). Also, the degree of oxidative stress is directly related to the inhibition of collagen production, and prolidase is considered as a target enzyme of this process⁽⁹⁾. This direct correlation has been shown in different physiologic and pathologic conditions⁽¹⁰⁾. The main reason behind the growth of prolidase activity is related to increased rates of collagen turnover indicative of extracellular matrix (ECM) remodelling⁽³⁾. Prolidase activity in fibroblast cultures of human skin was due to a rise in cell density, and increased cell density triggers increases in prolidase activity⁽¹¹⁾. Additionally, the prolidase activity was significantly increased in the lung fibrosis⁽¹²⁾.

A recent study demonstrated that, significant positive correlation between serum prolidase activi-

ty and fibrosis score was seen in patients with liver fibrosis⁽¹³⁾.

Prolidase activity may play a role in SSc pathogenesis due to the accumulation of collagen in tissues. However, the relationship between SSc and prolidase activity is unclear and has not been extensively studied. The aim of this study is to investigate serum prolidase enzyme activity in SSc patients and determine its relationship with serum oxidative status.

Materials and methods

Enrolment of study population

This study was conducted between October 2011 and May 2012 at the Department of Physical Therapy and Rehabilitation, Faculty of Medicine, Dicle University, Diyarbakir, Turkey. SSc patients (n = 21) and healthy volunteers (n = 29) were included in this study. Diagnoses were made according to the criteria of the American College of Rheumatology (ACR) 1980 criteria for the classification of scleroderma (14). Twenty-four patients diagnosed with SSc were invited to join the study. One patient was excluded due to lung cancer, and 2 patients were excluded due to diabetes mellitus. All the patients and control participants were female. The local ethics committee approved the present study. All the recruited subjects signed an informed consent form before participating into the study.

Clinical assessment

The demographic and clinical characteristics of the patients and current treatment methods were recorded (age; height; weight; disease duration, which is defined as the duration since the onset of the first symptoms of SSc; SSc subtype; organ involvement; cardiovascular conditions [Raynaud's phenomenon, digital ulcers]; gastrointestinal conditions [dysphagia, gastroesophageal reflux]; pulmonary conditions [dyspnea]; urogenital, nervous and musculoskeletal symptoms; and tender joint count [tender joint count was recorded using a 28-joint count]⁽¹⁵⁾. Other laboratory and clinical measurements conducted were as follows:

- The erythrocyte sedimentation rate (ESR) was measured through the Westergren method (mm/h).
- The serum C-reactive protein (CRP) level was determined with the help of nephelometry (mg/dl).
- The diffusing capacity of the lungs for car-

bon monoxide (DLCO) was assessed with the help of the pulmonary function test.

- Skin involvement was assessed using a modified Rodnan Skin Score (mRSS).

The mRSS clinically assesses the thickness/scleroderma of 17 body surface areas, giving each area a certain value on a scale of 0–3: 0=normal thickness; 1=mild thickness; 2=moderate thickness; 3=severe thickness (maximum score: 51) (16). In order to measure disease activity, the European League Against Rheumatism (EULAR) Scleroderma Trial and Research group criteria (or EUSTAR criteria) were used. EUSTAR criteria for disease activity include mRSS score >14 (scores 1), Scleredema (swelling and connective tissue; proliferation, particularly in the fingers) (scores 0-5); aggravation of skin involvement during the last month (scores 2); digital tip ulcerations (scores 0-5); aggravation of vascular complaints during the last month (scores 0-5); symmetric swelling and tenderness of peripheral joints (scores 0-5); DLCO <80% of expected value (scores 0-5); aggravation of heart or lung involvement during the last month (scores 2), ESR >30 (scores 1-5); and hypocomplementemia (low C3 or C4). A total score of ≥3 for all parameters indicates disease activity⁽¹⁷⁾.

Exclusion Criteria

Exclusion criteria included pregnancy; chronic disorders including coronary artery disease, diabetes mellitus, hypertension, dyslipidemia, and renal disease; malignancies, systemic or local infections; and a history of chronic alcohol consumption or of smoking in the past year.

Biochemical analyses

The fasting blood samples were immediately centrifuged at 4000 g for 10 min. Subsequently, the serum were transferred to an Eppendorf tube for storage at -50 oC until analysis. The TAS⁽¹⁸⁾ and TOS⁽¹⁹⁾ levels of serum were evaluated using automated methods developed by Erel. TAS results were expressed as mmol Trolox equivalent/L and TOS results were expressed as μ mol H₂O₂ equivalent/L. The percent ratio of TOS level to TAS level was accepted as the oxidative stress index (OSI)⁽²⁰⁾. The OSI value was calculated according to the following formula: OSI (Arbitrary Unit) = TOS (nmol H₂O₂ equivalent/L.) /TAS (nmol Trolox equivalent/L).

Serum paraoxonase-1 (PON-1) levels (U/L) were measured spectrophotometrically by modified

Eckerson method, and initial rates of hydrolysis of paraoxon (0.0-diethyl-(0-p-nitrophenyl) phosphate; Sigma Chemical Co. London, UK) were determined by measuring liberated p-nitrophenol at 405 nm at 37°C⁽²¹⁾.

Plasma prolidase levels (U/L) were determined via spectrophotometric method, which determines prolidase activity by measuring the proline levels produced by prolidase. The supernatant was diluted twofold with serum physiologic. Twenty-five microliters of the mixture were preincubated with 75 µL of the preincubation solution (50 mmol/L Tris HCl buffer pH 7.0 containing 1 mmol/L glutathione and 50 mmol/L MnCl₂) at 37°C for 30 min. The reaction mixture, which contained 144 mmol/L gly-pro, pH 7.8 (100 µL), was incubated with 100 µL of preincubated sample at 37°C for 5 min. To stop the incubation reaction, 1 mL glacial acetic acid was added. After adding 300 µL Tris HCl buffer, pH 7.8 and 1 mL ninhydrin solution (3 g/dL ninhydrin was melted in 0.5 mol/L orthophosphoric acid), the mixture was incubated at 90°C for 20 min and then cooled with ice. Absorbance was then measured at a 515 nm wavelength to determine proline by the method proposed by Myara et al.⁽²²⁾. This method is a modification of Chinard's method⁽²³⁾. The intra-assay and inter-assay coefficients of variation (CVs) were both lower than 7%.

Statistical analysis

All data were expressed as mean and standard deviation (SD). Kruskal–Wallis variance analysis, followed by a Mann–Whitney U-test, evaluated differences between groups. Relationships between variables were analysed by Pearson or Spearman correlation analysis according to the distribution type of the variables. P values <0.05 were considered statistically significant. All data were processed using the SPSS 18.0 for Windows (IBM Corporation, Armonk, NY) statistical package.

Results

The demographic, clinical, and laboratory data of the SSc patients and the control participants are shown in Table 1. The mean duration of SSc involvement was 4.94±4.36 years and the average modified Rodnan skin score was 15.70±6.76. There were no statistically significant differences between the two groups in terms of age. Higher serum CRP (p=0.002), and ESR (p=0.000) levels were observed

in SSc patients in comparison with the healthy participants.

	SSc (n=21)	Control (n=29)	P
Age (year)	40.83±13.12	34.25±6.47	0.076
Disease duration (year)	4.94±4.36	-	
Modified Rodnan skin score	15.70±6.76	-	
Mean hematocrit (%)	36.98±4.55	36.25±2.62	0.27
Mean CRP (mg/dl)	0.66±0.676	0.30±0.26	0.002
Mean ESR (mm/h)	23.61±14.00	8.76±4.37	0.000
Mean WBC (K/UL)	8.86±3.91	6.49±1.44	0.015

Table 1: Demographic, clinical and laboratory data of SSc and control groups, Mean ± SD.

CRP: C-reactive protein; **ESR:** Erythrocyte sedimentation rate; **WBC:** White blood count.

Serum prolidase enzyme activity (p=0.020), TOS (p=0.000), and OSI (p=0.000) were all significantly higher in BD patients. The serum TAS (p=0.063) and PON-1 (p=0.218) levels were lower in SSc patients, but this difference was not statistically significant. The serum prolidase activity, TAS, TOS, OSI, and PON-1 levels of the SSc patients and healthy participants are shown in Table 2. Serum prolidase enzyme activity was also significantly higher in Raynaud's phenomenon positive (n=18) SSc patients than Raynaud's phenomenon negative (n=3) SSc patients (p=0.025).

	SSc (n=21)	Control (n=29)	P
TAS (nmol Trolox equiv./L)	1.43±0.16	1.50±0.12	0.063
TOS (nmol H ₂ O ₂ equiv./L)	49.04±39.65	20.54±4.66	0.000
OSI	34.10±26.95	13.68±2.87	0.000
Prolidase (U/L)	89.52±39.33	70.60±17.60	0.020
PON-1 (U/L)	62.66±31.65	58.02±47.34	0.218

Table 2: Serum TAS, TOS, OSI, PON-1, and prolidase levels in SSc and control groups, Mean ± SD.

TAS: Total antioxidant capacity; **TOS:** Total oxidant status; **OSI:** Oxidative stress index; **PON-1:** Paraoxonase 1; **SSc:** Scleroderma.

No correlation existed between the serum proli-
dase activity and mRSS ($P=0.235$, $r=0.304$). A
negative significant correlation was observed
between Rodnan Skin Score and PON-1 levels
($r=0.526$, $P=0.03$). Serum TAS levels were positive-
ly correlated with Rodnan Skin Score ($P=0.008$,
 $r=0.623$) and duration of SSc involvement
($P=0.009$, $r=0.596$). There was no correlation
between the proli-
dase activity and serum oxidative
stress markers.

Discussion

Oxidative stress is a result of an increase in
reactive oxygen species (ROS) or an impairment of
antioxidant defense systems. ROS generated during
various metabolic and biochemical reactions have
multifactorial effects that include oxidative damage
to DNA, and can cause several abnormalities such
as endothelial cell damage. Additionally, ROS may
play a regulatory role in inflammation by modulat-
ing monocyte chemotactic activity. Thus, excessive
oxidative stress has been implicated in the patho-
genesis of SSc⁽²⁴⁾.

Recent studies have demonstrated the direct
role for ROS in the pathogenesis of SSc, including
auto-antibody and collagen production in mouse
model and human skin⁽²⁵⁾. Increased levels of lipid
peroxidation and decreased levels of antioxidant
capacity in SSc patients have been reported⁽²⁶⁾. On
the other hand, the level of superoxide dismutase,
one of the main components of antioxidant defence,
increase to a greater degree in SSc than in healthy
controls⁽²⁷⁾. TAS measurement in plasma from SSc
patients has been reported with conflicting results^{(28,}
²⁹⁾. TAS levels were found higher in patients with
SSc than in healthy controls^(28,29). However, TOS,
TAS, and OSI were not studied together in patients
with SSc in previous studies. In the present study,
we found that serum TAS levels were lower in SSc
patients but that the differences were not statistical-
ly significant. Serum OSI and TOS levels were sta-
tistically higher in the SSc groups than in the con-
trol group in our study. Additionally, serum TAS
levels positively correlated with Rodnan Skin Score
and with mean duration of SSc involvement.

The role of proli-
dase in the metabolism of col-
lagen is shown by some pathological conditions^{(7, 8,}
^{30, 31)}. The damage was supposed to be secondary to
the direct attack of proteoglycan and collagen mole-
cules by free radicals. Free radicals degrade colla-
gen, thereby preventing collagen from forming fib-

riils⁽³²⁾. Abraham et al. found higher plasma and liver
proli-
dase activity in an experimental rat liver fibro-
sis model⁽³³⁾. Duong et al. found a statistically sig-
nificant difference in proli-
dase activity in keloid tis-
sue as compared with normal skin tissues⁽³⁴⁾. It is
possible to argue that proli-
dase could be an impor-
tant marker in fibrotic diseases. As stated above,
proli-
dase activity might have a role in the patho-
genesis of SSc due to the accumulation of collagen.
However, we found no data in the literature related
to proli-
dase activity in patients with SSc. It has
found that the plasma proli-
dase activity was elevat-
ed in chronic inflammation⁽³⁵⁾.

In a study, the authors reported that the serum
proli-
dase enzyme activity was significantly higher
in the patients with Behçet's disease when com-
pared with healthy control subjects. Also in sub-
analysis of patients with Behçet's disease group,
serum proli-
dase enzyme activity was significantly
higher in active disease group than in patients
whose disease was inactive⁽³⁶⁾. In this study, signifi-
cantly higher serum proli-
dase activity was found in
patients with SSc compared with control partici-
pants.

The PON-1 enzyme is located on high-density
lipoprotein (HDL) and capable of hydrolysing oxi-
dized low-density lipoprotein (LDL). PON-1 also
protects HDL from oxidation⁽³⁷⁾. Due to this antioxi-
dant property of PON-1, HDL has antioxidant
activity and plays a protective role against athero-
genesis^(36,37). Ergüder et al.⁽³⁸⁾ found that HDL level
significantly decreased in the anticentromere anti-
body-negative SSc patients compared to control
groups. Furthermore, these same researchers⁽³⁸⁾ also
found that PON-1 activity in patients with SSc who
are anticentromere antibody-positive was signifi-
cantly higher than in patients who were antice-
ntromere antibody-negative or than in the control
group. In this study, the serum PON-1 levels were
higher in SSc groups than control participants but
were not statistically significant. In addition, a neg-
ative significant correlation was found between
Rodnan Skin Score and PON-1 levels.

All of our patients were using drugs to treat
SSc. These drugs included methylprednisolone,
colchicine, and calcium channel blockers, and these
drugs may impact oxidative status. However, we
did not ask our patients to stop taking their medica-
tions for extended periods of time before blood
sample collection. Patients were drug-free on the
day that the blood samples were drawn; however,
this is an inadequate amount of time for getting rid

of all drug effects. Although data is lacking on this topic, the potential effects of medications on serum prolidase activity, PON-1, TAS, TOS, and OSI should be kept in mind when evaluating our results.

This study is a preliminary investigation of prolidase activity in SSc patients, and some limitations must be taken into account. This study includes a limited number of patients and was not randomized. Importantly, SSc patients were accustomed to receiving certain therapies to manage their conditions. To further clarify these issues, prospective and randomized studies with larger samples are needed.

Conclusion

Serum prolidase enzyme activity and oxidative stress were both significantly higher in SSc patients in comparison to healthy controls. This data demonstrates a previously unreported association between serum prolidase enzyme activity and SSc. Increased prolidase activity may be associated with critical biological activities relevant to the pathology of SSc. However, randomized, controlled clinical studies are needed that include a larger number of patients.

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