Research article

Reduced serum paraoxonase-1 levels in vitiligo: further evidence of oxidative stress

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Vitiligo is a common disorder that results in depigmented areas of the skin. The pathogenesis of the disease remains unclear, but oxidative stress is one suggested cause. Oxidative stress may be induced by increasing the generation of reactive oxygen species and other free radicals. The generation of reactive oxygen species is known to be associated with a decrease in antioxidant levels. This study examined oxidative stress index in active lesions of generalized vitiligo patients. We analysed serum levels of paraoxonase 1, arylesterase, catalase, ceruloplasmin, total antioxidant capacity, and oxidative stress index in patients with active lesions of generalized vitiligo, as well as in matched, healthy controls. Serum oxidants and oxidative stress indexes were higher, and serum antioxidants were lower, in vitiligo patients compared with healthy controls. Our findings suggest that oxidative stress may play an important role in the pathogenesis of vitiligo. Paraoxonase 1 can be used as an indicator in determining oxidative stress existent in the pathogenesis of vitiligo diseases.

Keywords: Vitiligo, Paraoxonase 1, Oxidative stress index

Introduction

Vitiligo is an acquired skin disease with a global prevalence rate of 1–2% characterized by depigmented macules and patches that tend to be chronic and progressive.[1,2] All ages are affected and there are two main clinical types, segmental and non-segmental vitiligo.[3,4] Although essentially asymptomatic, the disease can be associated with considerable emotional distress and stigmatization.[5,6] The exact cause of vitiligo is unknown but the pathogenesis involves disappearance of functioning melanocytes in the basal layer, with resultant loss of melanin pigment.[2] Genetic and autoimmune factors have been implicated in aetiology, and there is also experimental evidence of oxidative stress in the disease.[2,7–9] Vitiligo has proven very challenging to treat, and current modalities offering partial improvement include phototherapy, topical corticosteroids and calcineurin inhibitors, and various surgical approaches. Oxidative stress was first noted in vitiligo from the presence of high levels of H_2O_2 in affected skin, and disturbed reactive oxygen species (ROS) homeostasis was demonstrated in tissue and blood of patients with vitiligo, especially when the disease was active.[2,10] ROS can damage key lipid, protein, and enzyme systems involved in melanogenesis, and they also impair protein-repair mechanisms.[2,11] Apart from direct or indirect evidence of elevated ROS in vitiligo patients, there is also evidence of deficient antioxidants.[4,7,11–20] The paraoxonase (PON) family of antioxidant enzymes degrade oxidized phospholipids, and have been shown to be deficient in many disease states. Polymorphisms of the PON gene are associated with the metabolic syndrome.[21] PON activity has not been reported in vitiligo before, and this study aimed to measure serum levels of PON1 in patients with active vitiligo, as well as matched controls. Arylesterase (ARE), catalase, and ceruloplasmin levels were also determined, as well as other parameters of oxidative stress like total oxidant capacity (TOC), total antioxidant capacity (TAC), and the oxidative stress index (OSI).

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Materials and methods

Subjects

The study included 35 patients with recent active lesions of generalized vitiligo (17 males and 18 females; median age 27.1 years; range 18–47 years)

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and 27 healthy volunteers as a control group (12 males and 15 females; median age 26.2 years; range 16–54 years). All patients had skin phototypes Fitzpatrick III–IV. The mean duration of the disease was 6.7 years in the vitiligo group. Blood samples were obtained from both the patient and control groups. PON1, ARE, CAT, ceruloplasmin, TAC, and TOC parameters were analysed in serum samples, and then, OSI was calculated. The study protocol followed the Declaration of Helsinki; all subjects were informed about the study protocol, and written consent was obtained from all participants or their guardians. The study was approved by the local Clinical Research Ethics Committee. The active phase of vitiligo was defined as the progression or appearance of new lesions within the previous 3 months.

Exclusion criteria

Exclusion criteria were the presence of chronic disease; concomitant inflammatory disease, such as infections and autoimmune disorders; immunocompromised state; diabetes mellitus; familial hypercholesterolemia; neoplastic diseases; liver or kidney diseases; and recent major surgical procedure. Untreated patients with active lesions of generalized vitiligo were examined.[22] Segmental vitiligo patients were excluded. Patients taking antioxidant drugs, vitamins, diuretics, or hormone replacement therapy, as well as smokers and those with alcoholism, were also excluded.

Measurement of paraoxonase 1 and arylesterase activities

PON1 and ARE activities were measured using paraoxon and phenylacetate substrates (Sigma Chemical Company, St Louis, MO, USA). The rate of paraoxon hydrolysis (diethyl-p-nitrophenyl phosphate) was measured by monitoring the increase of absorbance at 412 nm, at 37°C. The amount of generated p-nitrophenol was calculated from the molar absorption coefficient at pH 8, which was 17,000 $M^{-1}\,cm^{-1}$. PON1 activity was expressed as U/l serum. Phenylacetate was used as a substrate to measure ARE activity. The molar absorption coefficient of the produced phenol was 1310 $M^{-1}\,cm^{-1}$, and it was used to calculate enzymatic activity. One unit of ARE activity was defined as 1 μmol phenol generated/min under the above conditions and expressed as U/l serum. The PON1 phenotype distribution was determined by a double substrate method that measures the ratio of PON1 activity (with 1 M NaCl in the assay) to ARE activity, using phenylacetate.[23]

Measurement of total catalase levels

CAT activity was determined by Goth's colorimetric method (Sigma Chemical Company), in which supernatant was incubated in H_2O_2 substrate, and the enzymatic reaction stopped by the addition of ammonium molybdate. The intensity of the yellow complex formed by molybdate and H_2O_2 was measured at 405 nm.[24]

Measurement of total ceruloplasmin levels

Erel's ceruloplasmin measurement method was used (Sigma Chemical Company). This method is automated, colorimetric, and based on the enzymatic oxidation of ferrous ions to ferric ions.[25] The results were expressed in milligrams per decilitre, and the precision of this assay is within 3% (for details, see refs[25,26]).

Measurement of total antioxidant capacity

TAC levels of serum were determined using commercial Rel assay diagnostic kits (Gaziantep, Turkey) with an autoanalyser (Aeroset®, Abbott®, IL, USA) developed by Erel. Fe^{2+}-*o*-dianisidine complex with hydrogen peroxide by Fenton-type reaction generates to OH radicals. This powerful, reduced ROS reacts with colourless *o*-dianisidine molecules in low pH to form yellow-brown dianisidine radicals. Dianisidine radicals increase the formation of the colour by participating in an advanced oxidation reaction.

However, antioxidants that stop these oxidation reactions suppress the formation of the colour. The results are given by automated analysers used to measure this reaction spectrophotometrically at 240 nm. Trolox, a water-soluble vitamin E analogue, is used as a calibrator. The results were expressed as micromoles Trolox®. Equivalent per litre (μmol TE/L)[27].

Measurement of total oxidant capacity

TOC levels of serum were determined using commercial Rel assay diagnostic kits (Gaziantep, Turkey) with an autoanalyser (Aeroset®) developed by Erel. Oxidants oxidize the ferrous ion-o-dianisidine complex into ferric ion. Glycerol present in the media accelerates this reaction three-fold. Ferric ions form a coloured complex with xylenol orange in acidic media. The intensity of the colour, which is related to the amount of oxidants in the sample, is measured spectrophotometrically. H_2O_2 is used as a standard, and the results are expressed as μmol H_2O_2 equivalent/l.[28]

Measurement of oxidative stress index

The ratio of TOC to TAC gave the OSI, an indicator of the degree of oxidative stress. To perform the calculation, the TOC, expressed as μmol Trolox equivalent/L, was converted to μmol equivalent/L, and the OSI value was calculated by the formula: OSI = [TOC (μmol H_2O_2 Eq/L)/TAC (μmol Trolox Eq/L)] × 100.[25]

Statistical analysis

All analyses were conducted using the SPSS statistical program (version 11.5 for Windows; SPSS, Chicago, IL, USA). The normality of distributions was evaluated with the Kolmogorov–Smirnov test. Comparisons between patients and controls used the unpaired Student's *t*-test for normally distributed parameters and the Mann–Whitney *U*-test for non-normally distributed data. Results were expressed as mean ± standard deviation. All statistical tests were two-sided, and a P value ≤ 0.005 was considered significant.

Results

In Table 1, demographic findings of the vitiligo patients and the control group are presented. No statistically significant differences were found between the two groups in terms of age, sex, or body mass index. The mean serum PON1, ARE, CAT, and ceruloplasmin, and TAC levels were lower in the patient group than in the control group; the difference for PON1 ($P < 0.001$) was significant, whereas the other parameters showed non-significant differences (Table 2). The mean TOC level in serum and OSI were significantly higher in the vitiligo patients compared with the healthy controls ($P < 0.001$). No correlations were detected between PON1 and ARE, CAT, ceruloplasmin, TAC, TOC, or OSI.

Discussion

One of the major hypotheses in the pathogenesis of vitiligo is the oxidative stress hypothesis,[9,10] which is based on the reality of the formation of some toxic metabolites during melanin biosynthesis. Hydrogen peroxide formed at the end of all these alterations gives rise to the destruction of melanocytes by inhibiting CAT activity. Recently, an imbalance of oxidant/antioxidant associated with hydrogen peroxide accumulation and low CAT levels in the blood and epidermis of patients with vitiligo was reported.[2,4,7,10–20] Epidermal ROS sources may be endogenous, such as radicals formed due to activated neutrophils or enzyme activities, such as NADPH oxidase, xsantin oxidase, lipooxigenase, or nitric oxide synthases; it also may be exogenous, such as ultraviolet rays, which are prooxidative stimulants, atmospheric gases, microorganisms, pollution, and xsenobiotics.[2,29]

The balance between oxidative damage and antioxidant enzyme systems appears to determine the physiological and pathological effects of ROS.[30] These have the ability to interact with lipids, proteins, nucleic acids, and carbohydrates, and they may have damaging effects on biologic systems. The formation of oxidative stress was observed during the melanin biosynthesis of intermediator products that have direct toxic effects on melanocytes, such as 3,4-dihydroxyphenylalanine, dopachrome, and 5,6-dihydroxyindole.[31] It is considered that melanocyte destruction occurs in patients with vitiligo due to the accumulation of toxic compounds (H_2O_2) and inhibition of detoxification mechanisms (inhibits CAT activity), leading to oxidative stress.[2,8,13,32]

A broad antioxidant system present in the skin consists of an enzymatic and non-enzymatic antioxidant network. The enzymatic antioxidant system is constituted by glutathione peroxidase, CAT, and superoxide dismutase; intracellular non-enzymatic antioxidants consist of α-tocopherol, ubiquinone, β-carotene, ascorbate, and glutathione.[33] ROS are formed as an outcome of normal metabolism in healthy individuals, and is removed by antioxidants acting in the defence mechanism of the body. These processes work with a normal oxidant/antioxidant balance. Oxidative stress occurs when an imbalance towards the oxidant side occurs.[8,34]

Human serum PON1 and ARE are esterase enzymes with lipophilic features. Previous studies have revealed their role in ROS pathogenesis and the diminution of PON1 activity in oxidative stress, as well as in cases of inflammatory states, such as psoriasis and Behçet's disease.[35–39] PON1, an enzyme related to HDL, exhibits an antiatherosclerotic function by inhibiting LDL oxidation. Low PON1 activity has

Table 1 Demographic data of vitiligo patients and control group

Parameters	Vitiligo ($n = 35$)	Controls ($n = 27$)	*P* value
Age (years)	27.1 ± 9.4	26.2 ± 9.1	NS
Sex (M/F)	17/18	12/15	NS
BMI (kg/m2)	24.2 ± 3.3	23.5 ± 3,1	NS

Values are mean ± S.D.
BMI, body mass index; NS, not significant.

Table 2 Levels of PON1, CAT, ceruloplasmin, TAC, TOC, ARE, and OSI in vitiligo patients and control subjects

Parameters	Vitiligo ($n = 35$)	Controls ($n = 27$)	*P* value
Paraoxonase 1 (U/L)	108.70 ± 36.19	151.26 ± 40.35	0.001*
Arylesterase (kU/L)	139.95 ± 23.97	148.70 ± 35.83	NS
Catalase	7.92 ± 2.56	9.44 ± 1.47	NS
Ceruloplasmin (mg/L)	588.02 ± 64.90	604.56 ± 56.43	NS
TAC (μmol Trolox Eq./L)	0.89 ± 0.21	0.93 ± 0.13	NS
TOC (μmol H_2O_2 Equiv./L)	17.61 ± 5.55	10.44 ± 2.26	0.001*
OSI (AU)	2.07 ± 0.63	1.15 ± 0.31	0.001*

TAC, total antioxidant capacity; AU, arbitrary unit; NS, not significant.
*P values < 0.05 were considered statistically significant.

been demonstrated to be related to dyslipidaemia, diabetes mellitus, advanced age, smoking, hypertension, and increased oxidative stress. It has been verified that serum PON1 activity decreases significantly, and lipid peroxidase concentration increases significantly, in patients with metabolic syndrome.[36,39]

In vitiligo patients, we found lower levels of PON1 and ARE antioxidant enzymes, which prevent lipoprotein peroxidation and oxidation of LDL cholesterol. No correlations were observed between PON1 enzyme and other parameters. To our knowledge, these two enzymes have not been evaluated previously in vitiligo patients.

Sravani *et al.* detected low CAT levels in patients with vitiligo in comparison with a control group.[7] Other studies have shown low CAT serum levels in vitiligo patients.[10,34] We found lower serum CAT activity in vitiligo patients. We suggest that lower CAT activity may be associated with H_2O_2 accumulation, which may further inhibit CAT activity, thereby resulting in the destruction of melanocytes.[7,13]

Ceruloplasmin, an extracellular antioxidant that binds to free oxygen radicals, neutralises them by breaking their chains.[40] In the literature, studies displaying the levels of ceruloplasmin in patients with vitiligo are limited. Jain *et al.*[29] detected low ceruloplasmin levels in patients with generalized vitiligo. Boisseau-Garsaud *et al.*[40] detected normal ceruloplasmin levels in patients with vitiligo, compared with a control group. In our study, ceruloplasmin levels were low in generalized vitiligo patients compared with the control group, as reported by Boisseau-Garsaud *et al.* and Jain *et al.* Khan *et al.* reported lower serum TAC levels in vitiligo patients than in controls. However, the serum oxidative stress markers were high.[32] Other studies have reported a significant increase in serum oxidant levels with a decline in serum antioxidant levels in vitiligo patients.[10,40] In this study, vitiligo patients showed decreased serum TAC levels and increased serum TOC levels and OSI.

This finding indicates an imbalance in the oxidant/antioxidant system in the blood of vitiligo patients, and, thus, provides support for free-radical-mediated damage as a pathogenic event in vitiligo. We found that serum oxidants and OSI were higher, and serum antioxidants were lower, in vitiligo patients compared with healthy controls, and that the oxidative/antioxidative balance shifted to the oxidative side. Oxidative stress may play an important role in the pathogenesis of vitiligo. Melanocyte damage in vitiligo might be linked to oxidative stress. This is the first report on PON1 and ARE antioxidant parameters of vitiligo patients. The finding of a PON1 decrease in vitiligo patients emphasises the underlying hypothesis in the progression of the disease, and it can highlight the effect of free radicals and leading oxidative damage in vitiligo disease. However, further, larger studies are necessary to confirm our results.

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