Serum protein carbonyl and total antioxidant capacity levels in pemphigus vulgaris and bullous pemphigoid

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Background: Pemphigus diseases including pemphigus vulgaris (PV) and bullous pemphigoid (BP) are autoimmune diseases that cause severe blistering of the skin and mucous membranes. Among inflammatory mediators, reactive oxygen species (ROS) are involved in the pathogenesis of a wide variety of diseases through oxidative stress for which protein carbonyl (PC) and total antioxidant capacity (TAC) are two important markers.

Methods: We utilized PC and TAC in this study to compare the serum redox status of PV and BP patients with healthy subjects to investigate the possible role of oxidative stress in the pathogenesis of these diseases.

Results: The serum PC level was significantly higher in patients (3.07 ± 1.57 µM) than the healthy controls (0.86 ± 0.24 µM) ($P< 0.01$). The serum TAC was significantly higher in the patient group (3.45 ± 0.46 mM) compared with the healthy group (1.15 ± 0.05 mM) ($P< 0.01$). Besides, both serum PC and TAC levels showed no significant difference between PV and BP patients.

Conclusion: The findings of our study support the hypothesis that oxidative stress is involved in the pathogenesis of PV and BP.

Keywords: pemphigus vulgaris, bullous pemphigoid, protein carbonyl, total antioxidant capacity, oxidative stress

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INTRODUCTION

The outermost part of the body that is exposed to the surrounding environment is the skin which serves as the fundamental barrier of the immune defense system. This barrier provides protection against threats such as viruses and other microorganisms [1,2].

The outermost cellular tissue of the skin is the epidermis which produces several proteins that set up the essential protective tasks. Cytokines, prostaglandins, histamines, and chemokines are some inflammatory mediators which are expressed and secreted from keratinocytes which regulate the skin's immune responses [3,4].

Pemphigus vulgaris (PV) is an autoimmune

disease which results in severe blistering damage to the skin and mucous membrane [5-8]. PV is potentially fatal and could lead to malnutrition and sepsis [9,10]. Based on clinical, histopathological, and immunopathological criteria, autoimmune bullous diseases are classified into four major groups: pemphigus diseases, pemphigoid diseases, epidermolysis bullosa acquisita, and dermatitis herpetiformis (Duhring's disease) [11-13].

Pemphigus has a low incidence with a frequency of about 1 to 3.5 cases per 100,000 individuals annually worldwide [14]. It is more frequent amongst middle aged individuals. Research on PV, despite its low incidence, is useful for explanation of molecular pathogenesis of this type of autoimmune diseases and improvement of targeted therapies [14,15].

Oxidative stress induced by free radicals is considered one of the important molecular mechanisms involved in the pathogenesis of a wide variety of diseases [16-18]. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced by normal cellular metabolism in addition to their exogenous sources. This is a well-known fact that ROS and RNS have a dual role as both poisonous and beneficial species [19]. Physiological roles with advantageous effects of ROS occur at low or medium concentrations and include cellular responses such as defense against infectious microorganisms, a part of cellular signaling systems, and also the induction of a mitogenic response [20]. However, high concentrations of ROS have harmful effects, causing potential biological destruction which is termed oxidative stress [21-23]. Oxidative stress is an imbalance between oxidants and antioxidants in favor of the former [24,25]. The excessive ROS can oxidize and damage lipids, proteins, and DNA and inhibits their normal functions [26].

In an inflammatory environment, oxidative burst characterized by huge production of ROS has a key role in defense against pathogens. In this condition, activated neutrophils and macrophages produce enormous amounts of superoxide radical and other ROS via the phagocytic isoform of NAD(P)H oxidase [27].

On the other hand, since cell adhesion plays an important role in cell growth, embryogenesis, differentiation, wound repair, etc., the changes in the adhesive properties of cells and tissues are closely regulated by the redox condition [28,29].

According to these facts, we hypothesized that oxidative stress might play a role in the pathogenesis of blistering diseases with the assumption that oxidative stress produced in this autoimmune diseases could lead to disease symptoms such as blistering. In this investigation, we aimed to study the serum levels of protein carbonyl and total antioxidant capacity, as two important oxidative stress markers in two types of skin diseases including bullous pemphigoid (BP) and pemphigus vulgaris (PV), and compared them with the values in their normal counterparts.

MATERIALS AND METHODSS

Materials

The antioxidant assay kit (Item Number 709001) and Protein Carbonyl Assay Kit (Item Number 10005020) were purchased from Cayman Chemical Corporation, Ann Arbor, USA. All other chemical reagents were obtained from Sigma-Aldrich Company, Germany. The Perkin Elmer (Victor X5) plate reader was used for data measurement.

Sampling

Serum samples were obtained at the time of the diagnosis of PV or BP from the patients who had been admitted to the Department of Dermatology, Imam Reza and Ghaem Hospitals, Mashhad, Iran. Only patients who had not received any treatments were recruited for this study. The patients who had been treated with immune-suppressors and anti-inflammatory drugs or had used vitamin supplements or antioxidants in the last two months before sampling were excluded from the study. The control group was selected among healthy people who were matched in terms of age and gender. This study was approved by the Ethics Committee, Research Council of Mashhad University of Medical Sciences (code: 900395). Sampling was done after obtaining informed consent from the participants.

Sampling and preparation of samples were performed as follows: 5 ml blood was drawn from a brachial vein, collected into a dry tube and held at room temperature for 30 minutes. Thereafter, the serum was separated by centrifugation at 2000 rpm for 15 min, divided into aliquots, and stored at –80 °C until laboratory measurements.

Total Antioxidant Capacity

The Cayman Chemical antioxidant assay kit was used to measure the total antioxidant capacity of the serum samples. This method has been already described [30,31] and relies on the ability of antioxidants in the samples to prevent the oxidation of the 2,2′-Azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS) to ABTS•+. Under the reaction conditions, the serum antioxidants suppress the absorbance at 405 nm to a degree that is proportional to their concentration and activity. The capacity of the antioxidants in the serum sample to inhibit ABTS oxidation is compared against that of standard Trolox that is a water-soluble tocopherol analogue. Results are reported as micromoles of Trolox equivalent. This assay quantifies the collective antioxidant activities of the sample including vitamins, proteins, lipids, glutathione, and uric acid. All reagents and samples were equilibrated to room temperature before starting the assay. The duplicates Reagents and Trolox standards were prepared according to the manufacturer's recommendations. Then, 10 µL Trolox standard or sample was added to the corresponding wells of a 96-well plate. After that, 10 µL metmyoglobin and 150 µL chromogen were added to all standard and sample wells. The reaction was started by adding 40 µL hydrogen peroxide. After covering the plates and incubating them at room temperature for 5 minutes on a shaker, the absorbance was measured at 405 nm.

Protein carbonyls

The Cayman Chemical Protein Carbonyl Assay Kit was used in order to evaluate oxidized proteins based on a method described previously [32]. Some cations such as Fe^{2+} or Cu^{2+} can bind to the cation binding sites of the protein. This event, if accompanied by the attack of hydrogen peroxide or oxygen molecules, can convert amine groups on side-chains on arginine, histidine, lysine, and proline to carbonyl groups. In order to detect and evaluate the carbonyl amount in proteins, the assay was performed based on the reaction between protein carbonyls and 2,4-dinitrophenylhydrazin (DNPH). Protein carbonyls react with DNPH, forming Schiff base to produce the corresponding hydrazone. This product was analyzed spectrophotometrically at 380 nm.

Serum samples were analyzed according to the manufacturer's guidelines.

Statistical analysis

The data were analyzed using the SPSS 11.50 (SPSS Inc., Chicago, IL, USA) statistical software. Chi square and independent *t*-test were used for data analysis. A $P<0.05$ was considered statistically significant. Values were presented as means ± standard deviation (SD).

RESULTS

In this study, 15 patients comprised the case group and 15 healthy people composed the control group. The patients were divided into two groups based on their diagnosis of either pemphigus vulgaris (PV, n=9) or bullous pemphigoid (BP, n=6).

According to the result of chi-square test, the two groups had no significant difference in gender ($P<0.05$). Similarly, the chi-square test was used to investigate the gender difference between PV and BP patients. The result of the chi-square test showed no significant difference in gender between PV and BP patients ($P>0.05$).

Based on the result of the independent *t*-test, there was no significant difference in age between patients and healthy controls (64.47 ± 8.8 years *vs.* 61.80 ± 7.4 years, respectively; $P<0.05$).

Moreover, the Autoimmune Bullous Skin Disorder Intensity Score (ABSIS) [33] was used to investigate if our patients showed a difference in the severity of their diseases. Based on ABSIS, our patients were not significantly different (data not shown).

Comparison of serum total antioxidant capacity level between patients and controls

As shown in Figure 1.a, the total antioxidant capacity level was 3.45 ± 0.46 mM and 1.15 ± 0.05 mM in the serum samples of patients and healthy controls, respectively. Independent *t*-test showed a significant difference in the serum TAC level between the two groups ($P<0.01$). Besides, the serum TAC level was 3.25 ± 0.49 mM and 3.69 ± 0.43 mM in PV and BP patients, respectively. Independent *t*-test showed no significant difference between these two groups of patients (P>0.05) (Figure 1.b).

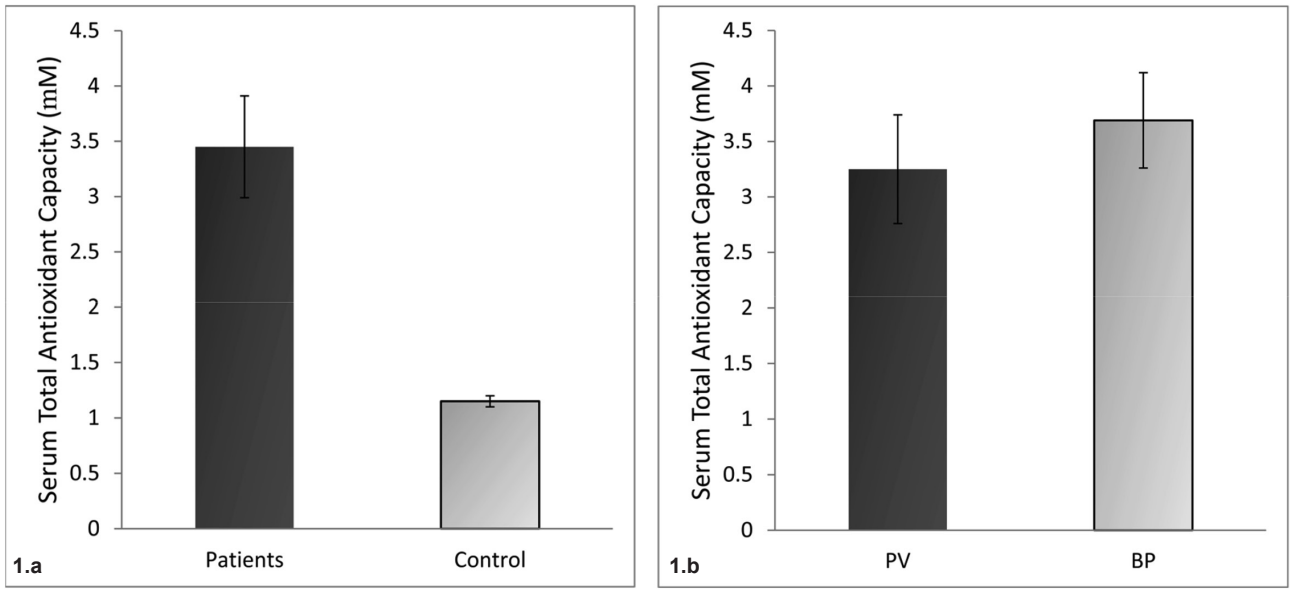

Figure 1. Serum Total Antioxidant capacity (TAC) was higher in patients than healthy subjects. Comparison of serum TAC values between patients and controls (**1.a**). The values of PV and BP patients are presented and compared (**1.b**).

Comparison of serum protein carbonyl level between patients and controls

The protein carbonyl level was 3.07 ± 1.57 µM and 0.86 ± 0.24 µM in the seum samples of patients and healthy controls, respectively (Figure 2.a). Independent *t*-test showed a significant difference in the protein carbonyl level between patients and controls ($P<0.01$).

Moreover, as shown in Figure 2.b, the serum protein carbonyl level was 3.23 ± 0.91 µM and 2.82 ± 1.76 µM in PV and BP patients, respectively. Independent *t*-test showed no significant difference between the two groups of patients in this regard ($P>0.05$).

DISCUSSION

In recent years, there has been a particular interest in investigating the relationship between

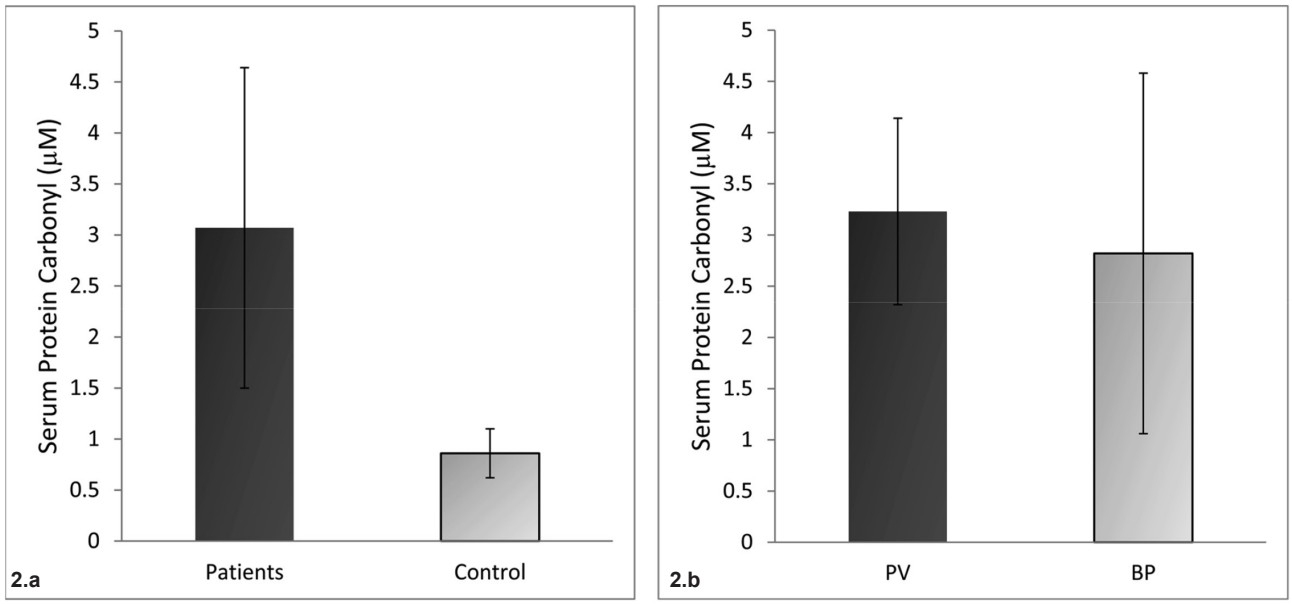

Figure 2. Serum protein carbonyl was higher in PV and BP patients compared to the control group (**2.a**). The difference between two groups of patients was not significant (**2.b**).

oxidative stress and inflammatory diseases. In this investigation, the protein carbonyl and total antioxidant capacity, as two important oxidative stress markers, were measured in the serum samples of BP and PV patients and compared with a healthy control group to evaluate any correlation between oxidative stress and pathogenesis of these autoimmune diseases.

Pemphigus is a distinctive skin-specific acquired autoimmune disease,which is induced by autoantibodies against desmosomal cadherins, desmoglein 1 (Dsg1), and Dsg3 [34-39].

Several studies have demonstrated these autoantibodies induce acantholysis within the mucous membranes and epidermis [37-39].

Important physiologic functions in the body, such as defense against pathogens, signal transduction, and apoptosis are performed by free radicals [40]. For example, there is a rise in ROS which leads to an oxidant-antioxidant imbalance in infections and inflammatory conditions. Furthermore, ROS can help with the production process of autoantibodies that subsequently help with triggering and maintaining the autoimmune cascade [41,42]. However, when there is excessive production of reactive oxygen species or insufficient elimination, they can lead to oxidative stress [40,43]. Thus, ROS may play a role in both the pathogenesis of autoimmune diseases and their progression and severity [42].

Based on the levels of serum protein carbonyl and total antioxidant capacity in PV and BP patients as well as in healthy persons as the control group, it was found that the balance of oxidant-antioxidant systems was disrupted in patients with the imbalance being in favor of oxidant levels.

We found that the protein carbonyl concentration was significantly higher in patients compared to the control group. However, there was no significant difference between BP and PV groups; these results signify an increase in free radical-mediated oxidation of cellular proteins and subsequent possible damage to the cell membrane in patients with PV and BP. This oxidative modification of cellular proteins may play a fundamental role in the physiopathology of PV and BP. It is well known that some cellular distresses are due to oxidized proteins that lose their natural structure and task [44,45]. For example, modified proteins represent potential targets for the immune system because these structural changes lead to the production of new epitopes, which results in an increased interaction between autoantibodies and autoantigens after oxidation [45,46]. Therefore, the protein carbonyl level might be used as an appropriate biomarker of oxidative stress in different types of autoimmune blistering diseases such as PV and BP.

Furthermore, the measurement of the total antioxidant capacity of our patients and healthy controls and the comparison of the obtained results revealed that the total antioxidant capacity increased in PV and BP patients. This result in turn suggests that in this group of diseases, increased oxidative stress has led to the activation of antioxidants to counteract this oxidative situation. However, this increase in antioxidant capacity was not enough to eliminate the harmful effects of oxidants, which led to oxidative stress.

Oxidative stress has recently become recognized as an important player in pathophysiology of several diseases, such as systemic lupus erythematosus, lichen planus, rheumatoid arthritis, and diabetes mellitus [47-52].

Alopecia is another example in which the role of oxidative stress has been increasingly examined in recent years. Alopecia is an autoimmune skin disease in which antibodies attack the hair follicles, leading to hair loss [53-56]. In general, alopecia patients have an oxidant- antioxidant imbalance in favor of oxidants, but the role of oxidative stress is not fully determined and it is not clear whether oxidative stress is the primary reason of the disease, or disease activity leads to an increase in oxidative stress.

The role of oxidative stress has also been investigated in vitiligo [57-60]. These studies support the idea that the oxidant-antioxidant balance disrupts in vitiligo in favor of oxidants. Similar to alopecia, it is unclear whether oxidative stress leads to the disease or vice versa.

Another skin disease in which the role of oxidative stress has been studied is psoriasis. Psoriasis is an autoimmune inflammatory skin disease due to the hyperproliferation of keratinocytes, and is characterized by inflammatory plaques on the skin. Some studies have shown an imbalance in the antioxidant-oxidant equilibrium between psoriatic patients and healthy controls [61,62]. These studies have confirmed a relationship between psoriasis and oxidative stress.

In conclusion, our findings support the

hypothesis that oxidative stress is involved in the pathophysiology of pemphigus. Oxidized proteins are targets for the immune system, leading to the production of antibodies that could explain the immunological feature of the disease and the role of inflammation in the onset and exacerbation of pemphigus and its acantholysis, the main feature of the disease. Further investigations are required to shed light on the exact role of oxidative stress in the physiopathology of pemphigus with a hope of finding new therapeutic protocols or using oxidative markers as prognostic factors.

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