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***Clinical Trials Study***

**Effect of anti-tumor necrosis factor-α antagonists on oxidative stress in patients with Crohn’s disease**

Yamamoto K *et al*. Oxidative stress and Crohn’s disease

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**Abstract**

**AIM**: To investigate changes in oxidative stress in Crohn’s disease (CD) before and after anti-tumor necrosis factor (TNF)-α treatment.

**METHODS**: A total of 42 patients with active CD, who were scheduled to be treated by anti-TNF-α antibodies, were enrolled. Serum levels of diacron-reactive oxygen metabolites (d-ROM), biological antioxidant potential (BAP), and modified ratio of oxidative stress and antioxidant capacity (m-OA) were measured using the Free Radical Analytical System before and eight weeks after induction therapy with infliximab or adalimumab. The values for oxidative stress were correlated with disease activity and clinical response as determined by the Crohn’s disease activity index (CDAI) at eight and 54 wk after the therapy.

**RESULTS**: Prior to treatment, d-ROM showed significant correlations with CDAI (*r =* 0.64, *P* < 0.01). There was a significant, negative correlation between m-OA and CDAI before and after treatment (*r =* -0.48 *vs* *r =* -0.42, *P* < 0.01). CDAI and d-ROM had decreased significantly by eight weeks after treatment (CDAI; 223.3 ± 113.2 *vs* 158.3 ± 73.4, *P* < 0.01, d-ROM; 373 ± 133 *vs* 312 ± 101, *P* < 0.05). However, neither BAP nor m-OA had changed significantly. In patients who had responded to the treatment at eight weeks, d-ROM, BAP and m-OA levels before treatment did not differ significantly between patients with and without loss of response.

**CONCLUSION**: Anti-TNF-α therapy decreases oxidative stress in patients with CD, but does not alter the production of antioxidants. Dysregulation of antioxidants may be associated with the disease.

**Key words:** Crohn’s disease; Severity; Oxidative stress; Anti-tumor necrosis factor-α antibody

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**Core tip:** We measured serum markers of oxidative stress (d-ROM) and antioxidant potential (BAP) prior to and eight weeks after the initial administration of infliximab or adalimumab in Crohn’s disease. As a consequence, d-ROM decreased significantly after the treatment. However, BAP and the ratio of oxidative stress and antioxidant potential remained unchanged. Anti-tumor necrosis factor-α therapy decreases oxidative stress, but does not alter the production of antioxidants. Dysregulation of antioxidants may be associated with Crohn’s disease.

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**INTRODUCTION**

Crohn’s disease (CD) is a chronic inflammatory condition of the intestinal tract. While the etiology is unknown, it is commonly thought that oxidative stress underlies the persistence of a chronic inflammatory process in the gut[1]. Oxidative stress is generally defined as an imbalance between pro-oxidant and anti-oxidant systems[2]. The oxidative stress consists of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Excessive ROS attack target molecules such as lipids, proteins and nucleic acids, and as a consequence can cause protein denaturation, inactivation of enzymes, and modifications to DNA[1,3,4]. Since CD is a disease characterized by neutrophil infiltration in the intestinal wall, there is a large degree of tissue damage due to the release of ROS[1,5].

To date, the influence of tumor necrosis factor (TNF) α, which is the most effective treatment for CD, on oxidative stress, has not been much investigated[6]. In 1999, Cesarone *et al*. described a simple procedure for measuring oxidative stress by means of quantification of serum diacron-reactive oxygen metabolites (d-ROM)[7]. d-ROM is a marker of hydroperoxide originating from ROS[8-11]. Biological antioxidant potential (BAP) is an antioxidant marker, which is representative of serum antioxidant capacity[12,13]. d-ROM and BAP can be readily measured by the Free Radical Analytical System 4 (FRAS4) (H&D srl, Parma, Italy)[13,14]. Furthermore, a modified ratio of oxidative stress to antioxidant capacity (m-OA), calculated from d-ROM and BAP, is believed to indicate the degree of resistance to oxidative stress.

In the present study, we measured d-ROM and BAP in CD patients before and after induction of remission by anti-TNF-α therapy to investigate whether oxidative stress is associated with disease activity and efficacy of anti-TNF-α antibodies. We also studied whether oxidative stress is associated with the long-term efficacy of the treatment.

**MATERIALS AND METHODS**

***Patients***

This was a pilot study of 42 patients with active CD, who were scheduled to be treated with anti-TNF-α antibodies. The demographics of the patients appear in Table 1. There were 30 males and 12 females with a mean age of 32 years. Eleven patients had ileal disease, 26 patients ileo-colonic disease, and five patients colonic disease. The duration of the disease ranged from 0 to 16 years with a mean of 6 years. Eight patients had a prior history of intestinal resection. Ten patients were treated with infliximab (IFX) while the remaining 32 patients were treated with adalimumab (ADA).

***Treatment and follow-up***

IFX was administered at a dose of 5 mg/kg intravenously at 0, 2 and 6 wk as induction therapy. Subsequently, IFX at the dose of 5 mg/kg every eight weeks was administered as maintenance therapy. ADA was administered subcutaneously at a dose of 160 mg at week 0 and 80 mg at week 2, followed by scheduled maintenance therapy at a dose of 40 mg every other week. Treatment responses were followed by physical examination and Crohn’s disease activity index (CDAI) scoring before and eight weeks after the initial administration of anti-TNF-α antibodies. Peripheral blood samples were also collected before and eight weeks after treatment. White blood cell count (WBC) and serum levels of CRP and albumin were measured. CDAI was assessed at eight weeks after treatment and every four weeks thereafter until 54 wk after the initial administration of anti-TNF-α.

***Assessment of disease activity***

Patients were assessed for response to treatment as defined by a decrease in the CDAI score of 70 points or more from the baseline value, and at least a 25% reduction in the total score after eight weeks of treatment. In patients who showed a clinical response to induction therapy, those who fulfilled any one of the following criteria were regarded as having a loss of response (LOR): (1) an increase in CDAI of at least 70 points from the qualifying score, with a total score of at least 175; (2) an increase in CDAI of 35% or more from the baseline value; or (3) the introduction of a new treatment for active Crohn’s disease[15].

***Measurement of oxidative stress***

Oxidative stress was measured as serum d-ROM, BAP and m-OA. The stress was assessed before and eight weeks after the initial administration of anti-TNF-α. Blood samples were stored on ice after collection, and centrifuged to separate the serum. The serum was then stored at -80 °C until analysis.

Immediately prior to measurement, the samples were dissolved in room temperature and puddle in a vortex mixer. To measure ROM levels, the FRAS4 system was used. For the measurement of d-ROM, 20 µL serum and 1mL buffered solution (R2 reagent of the kit, pH 4.8) were mixed in a cuvette, and then 10µL chromogenic substrate (R1 reagent) was added. After mixing and centrifugation for 60 seconds, the cuvette was incubated in a thermostatic block for 5 min at 37 °C. After that, the absorbance at 505 nm was recorded. Measurements were expressed as Carr U. Reference values measured by the manufacturer were indicated as being from 250 to 300 Carr U. Values greater than 300 Carr U suggest oxidative stress[7-9].

To measure BAP levels, the BAP test was performed using the same analyzer. In brief, 50 µL R2 reagent (ferric chloride) was added to a cuvette containing R1 reagent (thiocyanate derivative). The absorbance was measured to read and subtract the reagent blank value. Then, 10 µL serum sample was added to the cuvette. After incubation for 5 min at 37 °C, the absorbance at 505 nm was recorded. The BAP levels were expressed as μmol/L. Reference values provided by the manufacturer were greater than 2200 μmol/L. Values lower than 2200 μmol/L suggest a reduction of antioxidant capacity[8,12]. The modified ratio of oxidative stress to antioxidant capacity (m-OA) was calculated as BAP/d-ROM/7.541[16].

***Statistical analysis***

Data were expressed as mean (SD), or as median (25th-75th percentiles) when appropriate. Values were compared between groups using the paired *t*-test or Student’s *t*-test, as appropriate. Correlation coefficients were assessed by linear regression analysis. *P* < 0.05 was considered to be statistically significant.

**RESULTS**

***Efficacy of anti-TNF-α antibody***

After eight weeks, 32 (76%) of the patients showed a response to the treatment. At 54 weeks, 22 patients (52%) were in remission, while six patients (14%) had dropped out, and four (15%) had shown LOR. CDAI had decreased significantly eight weeks after treatment (223.3 ± 113.2 *vs* 158.3 ± 73.4, *P* < 0.01). Changes in serum albumin, white blood cell count and CRP are shown in Table 2. CRP values decreased significantly, while there was no statistically significant change in serum albumin or white blood cell count.

***d-ROM, BAP and m-OA levels***

Figure 1 illustrates changes in d-ROM, BAP and m-OA before and eight weeks after the induction therapy. After eight weeks, d-ROM had decreased significantly (373 ± 133 *vs* 312 ± 101, *P* < 0.05) (Figure 1A). The decrease was statistically significant in each type of regional involvement (ileal, ileocolonic, or colonic disease) (data not shown). However, neither BAP nor m-OA had changed significantly after eight weeks (Figure 1B, 1C).

***Correlations between oxidative markers and clinical parameters***

Table 3 shows the correlations between oxidative markers and clinical parameters. Before treatment, d-ROM showed statistically significant correlations with CRP (*r =* 0.64) and CDAI (*r =* 0.42) (Figure 2A). The correlation between d-ROM and CRP was significant even after the induction therapy (*r =* 0.53). d-ROM showed no significant correlation with the other parameters either before or after treatment. We failed to find any significant correlation of BAP with CRP, CDAI, WBC or serum albumin.

There were statistically significant negative correlations between m-OA and CDAI both before (*r =* -0.48) and after (*r =* -0.42) treatment (Figure 2B, 2C). There were negative correlations between m-OA and CRP before (*r =* -0.63) and after (*r =* -0.45) treatment. Significant correlations were also observed between d-ROM and CRP. Furthermore, we found a positive correlation between m-OA and serum albumin before (*r =* 0.45) and after (*r =* 0.47) treatment.

***Correlation between efficacy of anti-TNF-α and oxidative stress***

We then compared d-ROM, BAP and m-OA between responders and non-responders after eight weeks of treatment. d-ROM, BAP and m-OA were not significantly different between the two groups of patients either before or after treatment.When we compared d-ROM, BAP and m-OA before and after the therapy between patients with and without LOR, we found no significant differences, suggesting that these parameters of oxidative stress are not predictive of LOR.

**DISCUSSION**

Increases in oxidative stress have been demonstrated in the serum and intestinal mucosa of patients with CD[17-20]. More recently, Kupcova *et al*[21] described a reduction of oxidative stress in the mucosa of patients with CD after administration of anti-TNF-α antibody. In the present investigation, we found that serum oxidative stress decreased in patients with CD after anti-TNF-α treatment. We also showed that the ratio of antioxidant activity *vs* oxidative stress calculated as m-OA was negatively associated with the activity of CD even after anti-TNF-α treatment. These findings strongly suggest that a decrease in antioxidant activity is characteristic of CD, and that m-OA may be one of the specific biomarkers for patients with the condition.

d-ROM and BAP levels have been used to assess oxidative status, and their significance as clinical markers has been described in several fields. The two parameters have been studied particularly in cardiovascular disease, central nervous system disease and lifestyle-related illnesses, and it has been shown that increases in reactive oxygen metabolites are associated with an increased risk of vascular endothelial damage[22-26]. Furthermore, Sugiura *et al*[22] measured serum d-ROM levels in patients at a high risk of cardiovascular diseases and found that d-ROM level coupled with serum high-sensitivity CRP was predictive of cardiovascular events. In our patients with CD, we found that d-ROM, serum CRP and CDAI decreased significantly during the induction. Although we have not presented the data, there was no difference in the decrease of d-ROM between patients treated with IFX and those with ADA. Thus it seems likely that anti-TNF-α therapy suppresses the production of oxidative stress, thereby resulting in clinical benefit. In consideration of the biologic activity of anti-TNF-α, a decrease in the activity of circulating neutrophils seems to have made an important contribution to the decrease in oxidative stress measured as d-ROM.

Unlike d-ROM, we failed to show any changes in BAP, which represents antioxidant capacity, in our patients with CD even after anti-TNF-α treatment. While we did not include healthy controls in this investigation, the values of BAP in our patients were generally low, with more than half of the patients having values less than 2200 μmol/L. In previous studies, a reduction of serum antioxidant capacity was found in patients with inflammatory bowel disease (IBD)[17,27,28]. On the other hand, evidence for enhancement of antioxidant capacity was also found in biopsy specimens from IBD patients[19]. However, since the latter investigation included both patients with ulcerative colitis and those with CD, it still uncertain that the antioxidant capacity was actually increased in CD. From our present results, it seems likely that the impairment of antioxidant capacity is specific to CD. This may be a consequence of a genetically determined disturbance in autophagy, which does not seem to be increased by any therapeutic strategy[29].

Normally, cells handle oxidative stress by mechanisms that include the production of antioxidant agents. These compounds have a limited capacity to buffer against an increase in oxidative stress and prevent its toxic consequences. There are several naturally occurring antioxidant defenses in the human intestine that serve to protect cell membranes from lipid peroxidation, protein oxidation, and enzyme inactivation[18]. In this regard, m-OA has been considered to be a candidate serum marker for the balance between oxidative stress and antioxidant capacity. In the present investigation, m-OA showed the strongest and negative correlations with CDAI and CRP. Furthermore, the correlations were significant even after induction of ant-TNF-α antibody treatment. These observations strongly suggest that m-OA is a marker of persistent chronic inflammation of the intestine, and may be one of the surrogate biomarkers for patients with CD. While we could not show any significant utility of m-OA for predicting the short-term response to anti-TNF-α treatment or LOR in our CD patients, the potential role of the marker warrants further study.

There are some limitations to this study. First, as we used frozen samples of serum, we may have underestimated the concentrations of d-ROM, BAP and m-OA. Second, as we did not enroll healthy controls, we could not determine the reference values of the concentrations of the markers for oxidative stress, especially BAP, in the Japanese population. Third, since our investigation was a pilot study with relatively a small number of patients, we could not make certain conclusions regarding the roles of d-ROM, BAP and m-OA in the prediction of the response to treatment or the long-term clinical course of CD. This was especially the case for the lack of data regarding serum concentrations of and serum antibodies to IFX and ADA. Furthermore, we could not apply practical intestinal damage, namely mucosal healing, as a parameter of disease activity[30]. These issues are under investigation in another prospective study.

In conclusion, we have shown that anti-TNF-α therapy decreases oxidative stress in patients with CD without affecting the production of antioxidants. In addition, m-OA, a marker for the balance between oxidative stress and antioxidant capacity, showed significant correlations with other parameters of disease activity in CD. Therefore, we propose that impairment of antioxidant activity might be associated with intractability of CD.

**COMMENTS**

***Background***

While the etiology of Crohn’s disease (CD) is unknown, it is commonly thought that oxidative stress underlies the persistence of a chronic inflammatory process in the gut.

***Research frontiers***

We investigated whether oxidative stress is associated with disease activity and efficacy of anti- tumor necrosis factor (TNF)-α antibodies in patients with CD.

***Innovations and breakthroughs***

Serum oxidative stress decreased after anti-TNF-α treatment, while antioxidant capacity remained unchanged. The ratio of antioxidant activity *vs* oxidative stress (m-OA) negatively correlated with disease activity prior to and after anti-TNF-α treatment. Dysregulation of antioxidants may be associated with CD.

***Applications***

The ratio of antioxidant activity *vs* oxidative stress measured by m-OA may be one of the specific biomarkers for patients with CD.

***Peer-review***

This paper is interesting to know a relationship between oxidative stress and Crohn’s disease. The authors have necessary additional investigation in order to adjust for certain confounder in the correlation with oxidative markers.

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A

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B

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C

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**Figure 1 Changes in oxidative stress markers before and eight weeks after anti-TNF-α treatment in patients with Crohn’s disease.** A: d-ROM decreased significantly eight weeks after the treatment; B: BAP did not change significantly after anti-TNF-α treatment; C: m-OA did not change significantly after anti-TNF-α treatment. TNF: Tumor necrosis factor; m-OA: Modified ratio of oxidative stress and antioxidant capacity; BAP: Biological antioxidant potential; d-ROM: Diacron-reactive oxygen metabolite; NS: Not significant.

A

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B

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C

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**Figure 2 Correlation of d-ROM and m-OA with Crohn’s disease activity index.** A: d-ROM showed a significant correlation with CDAI before anti-TNF-α treatment (*r* = 0.42, *P* < 0.01); B: and C: m-OA showed significant correlations with CDAI both before (B) and after (C) the induction therapy. m-OA: Modified ratio of oxidative stress and antioxidant capacity; d-ROM: Diacron-reactive oxygen metabolite; CDAI: Crohn’s disease activity index.

**Table 1 Clinical characteristics of the patients *n* (%)**

|  |  |
| --- | --- |
| **Characteristics** | **All patients (*n* = 42)** |
| Gender | Male | 30 (71)12 (29) |
|  | Female |
| Age (yr), median | 31.9 (17-57) |
| Disease duration (yr), median | 6.2 (0-16) |
| Disease type |  | 11 (26)26 (62)5 (12) |
|  | Ileal |
|  | Ileo-colonic |
|  | Colonic |
| Patients with concomitant medication | 41 (98)9 (21)7 (17)24 (57)8 (19) |
|  | 5-aminosalicylic acid |
|  | Prednisone |
|  | Azathioprine |
|  | Enteral nutrition |
| Previous segmental resection |
| Infliximab | 10 (24) |
| Adalimumab | 32 (76) |

**Table 2 Biochemical tests for C-reactive protein, albumin, and white blood cell**

|  |  |  |  |
| --- | --- | --- | --- |
| Biochemical tests | Before | 8 wk after | *P* value |
| CRP (mg/dL) | 2.4 ± 2.6 | 1.1 ± 1.6 | < 0.01 |
| Alb (g/dL) | 3.4 ± 0.75 | 3.7 ± 0.84 | NS |
| WBC (per μL)  | 7339 ± 2484 | 7278 ± 2532 | NS |

CRP: C-reactive protein; WBC: White blood cell; Alb: Albumin; NS: Not significant.

**Table 3 Correlations between d-ROM, BAP, m-OA, CDAI, serum CRP, WBC, and serum albumin: All Crohn’s disease patients for treatment by anti-TNF-α**

|  |  |  |  |
| --- | --- | --- | --- |
| Oxidative markers | Clinical parameters | *r* | *P* value |
| d-ROM | CDAI | Before | 0.420.330.640.530.120.03-0.33-0.38 | < 0.010.03< 0.01< 0.010.470.840.030.01 |
|  | After |
|  | CRP | Before |
|  | After |
|  | WBC | Before |
|  | After |
|  | Alb | Before |
|  | After |
|  |  | -0.17-0.180.030.12-0.040.010.240.14 | 0.280.240.850.440.790.950.130.38 |
| BAP | CDAI | Before |
|  | After |
|  | CRP | Before |
|  | After |
|  | WBC | Before |
|  | After |
|  | Alb | Before |
|  | After |
|  |  |  |  |
| m-OA | CDAI | Before | -0.48-0.42-0.63-0.45-0.20-0.010.450.47 | < 0.01< 0.01< 0.01< 0.010.210.73< 0.01< 0.01 |
|  | After |
|  | CRP | Before |
|  | After |
|  | WBC | Before |
|  | After |
|  | Alb | Before |
|  | After |

TNF: Tumor necrosis factor; m-OA: Modified ratio of oxidative stress and antioxidant capacity; BAP: Biological antioxidant potential; d-ROM: Diacron-reactive oxygen metabolite; CDAI: Crohn’s disease activity index; CRP: C-reactive protein; WBC: White blood cell; Alb: Albumin; NS: Not significant.