Peripheral lymphocyte DNA damage and oxidative stress in patients with ulcerative colitis

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INTRODUCTION
Ulcerative colitis (UC) is a fairly common chronic inflammatory disorder. Chronic inflammation may contribute to the risk of colorectal cancer through the accumulation of specific products resulting from DNA damage. Previous studies reported that DNA damage and oxidative stress play a significant role in the pathophysiology of UC, but the results are inconsistent.

OBJECTIVES
In the present study, we investigated peripheral DNA damage and oxidative stress in patients with UC.

PATIENTS AND METHODS
The study included 20 patients with UC and 20 controls. Peripheral lymphocyte DNA damage was measured using the alkaline comet assay. Plasma total antioxidant capacity (TAC), total oxidant status (TOS), and oxidative stress index (OSI) were determined.

RESULTS
DNA damage levels, TOS, and OSI were significantly higher in patients with UC than in controls (P <0.001 for all parameters), while TAC was significantly lower (P <0.001). DNA damage was significantly correlated with TOS, TAC, and OSI (r = 0.604, P <0.001; r = –0.593, P <0.001; and r = 0.716, P <0.001, respectively). Moreover, TAC levels were significantly correlated with TOS and OSI (r = 0.604, P <0.001; r = –0.399, P <0.05; and r = –0.513, P <0.05, respectively).

CONCLUSIONS
Our results show that increased peripheral DNA damage and oxidative stress seem to be associated with decreased antioxidant levels and thus may in part contribute to the development of colorectal cancer associated with UC.
Previous studies have shown that oxidative stress causes an increase in the levels of proinflammatory cytokines.\(^4,5\) During chronic inflammation, when sustained production of reactive oxygen species (ROS) occurs, antioxidant defenses can weaken, resulting in oxidative stress.\(^5\) Increased production of reactive oxygen and nitrogen species has been observed after in vitro stimulation of the whole colon mucosa, mucosal macrophages, and peripheral blood monocytes of IBD patients.\(^5,7\) Antioxidant enzymes are known to prevent the promotion of carcinogenesis. It has been suggested that a defect in mucosal antioxidant defenses is an etiological factor in IBD.\(^8,9\)

Chronic inflammation has long been recognized to be associated with increased risk of human cancer at various sites, for example UC with the risk of colorectal cancer.\(^10,11\) With long-standing UC, dysplastic changes occur frequently and a dysplasia-carcinoma sequence is generally accepted.\(^13\) Oxidative stress can arise when the production of ROS exceeds the cellular antioxidant capacity, resulting in damage to cellular macromolecules such as DNA, proteins, and lipids.\(^14\) Oxidative stress has been shown to play a significant role in inflammation-associated carcinogenesis via DNA damage.\(^5\) Also, several studies have suggested that genomic damage plays a major role in the number of chronic diseases and processes, including various cancers, cardiovascular and neurodegenerative diseases, inflammation/infection, and aging.\(^16,17\) Oxidative stress resulting in DNA damage is considered to be the most common insult affecting the genome.\(^18\)

It is generally thought that DNA damage in lymphocytes can reflect the level of oxidative stress in the body.\(^19\) The comet assay was originally developed to measure DNA strand breaks with high sensitivity.\(^19\) Previous studies have shown that DNA damage is significant in the pathophysiology of UC,\(^19,20\) but the results are controversial. Furthermore, there are no data concerning peripheral DNA damage and oxidative stress in UC patients. Therefore, we aimed to investigate oxidative stress and DNA damage level in peripheral blood lymphocytes using the comet assay in this patient group.

**PATIENTS AND METHODS** The study involved 20 patients with UC (11 with active disease and 9 in remission) and 20 controls. Patients with active form had a documented diagnosis of UC. At the time of the study, these patients experienced symptoms of bloody diarrhea, urgency, and cramps and demonstrated typical sigmoidoscopic and histologic features of active UC as described previously.\(^21\) Patients with inactive UC had a documented history of active UC but were currently symptom-free. They had normal mucosa on sigmoidoscopy and histological features typical of inactive UC. All subjects consumed their usual diet at the time of the study. Average follow-up for patients with UC was 10.2 ± 2.3 months.

Diagnosis of UC was established on the basis of clinical, radiologic, endoscopic, and histopathological findings. The disease activity for UC patients was assessed as described by Rachmilewitz.\(^22\) In addition, an increase in C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR), platelet and leukocyte counts, and a decrease in hemoglobin and serum albumin levels were considered as indicative of disease activity.\(^23\) Patients with UC were allowed to use mesalazine or sulfasalazine, but these medications were stopped 24 hours before sigmoidoscopy.

Patients with UC were excluded if they had a history of alcohol abuse, smoking habit, intravenous drug abuse, pregnancy, micronutrient supplements, fish-oil supplement in the previous month, cryoglobulinemia, human immune deficiency virus infection, active infection, hypertension, diabetes, hyperlipidemia, chronic respiratory insufficiency, rheumatoid arthritis, cirrhosis, renal disease, coronary heart disease, cerebrovascular disease, or malignant tumor(s).

Control subjects were free of gastrointestinal symptoms and had normal colonoscopy. Control subjects were excluded if they had a history of alcohol abuse, smoking habit, intravenous drug abuse, pregnancy, micronutrient supplements, fish-oil supplement in the previous month, cryoglobulinemia, human immune deficiency virus infection, active infection, hypertension, diabetes, hyperlipidemia, chronic respiratory insufficiency, rheumatoid arthritis, cirrhosis, renal disease, coronary heart disease, cerebrovascular disease, or malignant tumor.

The study was conducted according to the Helsinki Declaration as revised in 1989 and was approved by the local ethics committee. All subjects provided their written informed consent to participate in the study.

**Measurement of the total antioxidant capacity** Plasma total antioxidant capacity (TAC) levels were determined using an automated method developed by Erel.\(^24\) The results are expressed as mmol Trolox equiv./l.

**Measurement of the total oxidant status** Plasma total oxidant status (TOS) levels were determined using a novel automated method developed by Erel.\(^25\) The results are expressed as μmol H₂O₂ equiv./l.

**Calculation of the oxidative stress index** The oxidative stress index (OSI) was defined as the ratio of the TOS to TAC levels.\(^26\) OSI (arbitrary unit) = TOS (μmol H₂O₂ equiv./l) / TAC (mmol Trolox equiv./l).

**DNA damage determination by the alkaline comet assay** After an overnight fasting, 6 ml of peripheral blood sample from each subject was withdrawn into a heparinized tube, kept on ice and lymphocyte isolation for the comet assay performed within 2 hours as described elsewhere.\(^27\)
**TABLE 1** Demographic and clinical data in obese subjects and controls

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Controls (n = 23)</th>
<th>Obese subjects (n = 25)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>age, y</td>
<td>26 ± 3</td>
<td>28 ± 2</td>
<td>NS</td>
</tr>
<tr>
<td>sex (women/men)</td>
<td>10/13</td>
<td>14/11</td>
<td>NS</td>
</tr>
<tr>
<td>body mass index, kg/m²</td>
<td>21.5 ± 1.87</td>
<td>32.21 ± 1.34</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>glucose, mmol/l</td>
<td>5.22 ± 0.22</td>
<td>5.38 ± 0.11</td>
<td>NS</td>
</tr>
<tr>
<td>triglycerides, mmol/l</td>
<td>1.52 ± 0.24</td>
<td>2.01 ± 0.42</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>TC, mmol/l</td>
<td>3.68 ± 0.67</td>
<td>4.54 ± 0.81</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>HDL-C, mmol/l</td>
<td>1.37 ± 0.27</td>
<td>0.89 ± 0.15</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>LDL-C, mmol/l</td>
<td>1.61 ± 0.55</td>
<td>2.73 ± 0.69</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Values are presented as mean ± standard deviation.

Abbreviations: HDL-C – high-density lipoprotein cholesterol, LDL-C – low-density lipoprotein cholesterol, NS – nonsignificant, TC – total cholesterol.

The endogenous DNA damage in lymphocytes was analyzed by the alkaline comet assay according to Singh et al.,\(^{28}\) with a minor modification. Thus, lymphocytes were embedded in 0.7% low melting point agarose in phosphate buffer saline at 37°C at the final concentration of 104 cells/ml. Then, 80 µl of this cellular suspension was spread on roughened slides that had previously been coated with 1.0% hot (60°C) normal melting point agarose, covered with a cover slip at 4°C for at least 5 minutes to allow the agarose to solidify, and transferred to a humidified box after removal of the cover slips. The slides were allowed again to solidify for 5 minutes at 4°C in a moist box. After removing the cover slips, the slides were submersed in freshly prepared cold (4°C) lysing solution (2.5M NaCl, 100mM EDTA-2Na; 10 mM Tris–HCl, pH 10–10.5; 1% Triton X-100 and 10% dimethyl sulfoxide (DMSO) added immediately before use) for at least 1 hour. The slides were then immersed in freshly prepared alkaline electrophoresis buffer (0.3 mol/l NaOH and 1 mmol/l Na2EDTA, pH >13) at 4°C for unwinding (40 min) and then they were subjected to electrophoresis (25 V/300 mA, 25 min). All of the above steps were conducted under red light and without direct light in order to prevent additional DNA damage. After electrophoresis, the slides were stained with ethidium bromide (2 µ/ml in distilled H₂O; 70 µl/slide), covered with a cover slip and analyzed using a fluorescence microscope (Nikon). Images of 50 randomly selected cells (25 cells from each of the two replicate slides) were analyzed visually from each subject as described elsewhere.\(^{27,28}\) Each image was classified according to the intensity of the fluorescence in the comet tail and was given a value of either 0–3 or 4 (from class 0 [no damage] to class 4 [maximum damage]), so that the total scores of slide could be between 0 and 200 arbitrary units. All procedures were completed by the same staff who was not aware of the diagnosis.

**Statistical analysis** The results were expressed as mean ± standard deviation. Qualitative variables were assessed by the χ² test. Parametric variables were compared using the Student t test. Correlation analyses were performed using the Pearson’s correlation test. P <0.05 was considered statistically significant. The analysis was performed with the SPSS 11.0 statistical software.

**RESULTS** The demographic, clinical, and laboratory data of patients with UC and controls are presented in **Table 1**. There were no statistically significant differences between patients with UC and the control group with respect to age, sex, and body mass index (P >0.05). In addition, the average follow-up for patients with UC was 10.2 ±2.3 months.

ESR, CRP levels, albumin levels, as well as platelet and leukocyte counts were higher in patients with UC than in controls (P <0.05, P <0.05, P <0.05, P <0.05, and P <0.01, respectively) (**Table 1**). Peripheral lymphocyte DNA damage, TOS levels, and OSI values were significantly higher in patients with UC than in controls (P <0.001), while TAC levels were significantly lower (P <0.001) (**Table 2**). When patients with UC were divided according to disease activity, no differences were observed between active UC and inactive UC with respect to peripheral lymphocyte DNA damage, TOS levels, TAC levels, and OSI values (P >0.05).

Peripheral lymphocyte DNA damage was significantly correlated with TOS levels, TAC levels, and OSI values (r = 0.604, P <0.001; r = –0.593, P <0.001, r = 0.716, P <0.001; respectively). In addition, TAC levels were significantly correlated with TOS levels and OSI values (r = –0.604, P <0.001; r = –0.399, P <0.05; respectively).

We observed that peripheral lymphocyte DNA damage in UC patients was significantly correlated with CRP levels, ESR, albumin levels, and platelet and leukocyte counts (r = 0.544, P <0.001; r = 0.725, P <0.05; r = –0.357, P <0.05; r = 0.525, P <0.001; r = 0.655, P <0.001; respectively). TAC was significantly correlated with CRP, ESR, albumin levels, platelet and leukocyte counts (r = –0.719, P <0.001; r = –0.686, P <0.05; r = 0.307, P <0.05; r = 0.504, P <0.001; r = 0.577, P <0.001; respectively).

In patients with UC, TOS and OSI were significantly correlated with CRP (r = 0.416,
Values are presented as mean ± standard deviation

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Patient group (n = 20)</th>
<th>Control group (n = 20)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>total antioxidant capacity, mmol Trolox equiv./l</td>
<td>0.92 ± 0.41</td>
<td>1.35 ± 0.12</td>
<td>0.001</td>
</tr>
<tr>
<td>total oxidant status, μmol H₂O₂ equiv./l</td>
<td>7.84 ± 1.79</td>
<td>5.11 ± 1.39</td>
<td>0.001</td>
</tr>
<tr>
<td>oxidative stress index, arbitrary unit</td>
<td>0.63 ± 0.16</td>
<td>0.34 ± 0.11</td>
<td>0.001</td>
</tr>
<tr>
<td>DNA damage, arbitrary units</td>
<td>69.90 ± 13.89</td>
<td>30.20 ± 8.70</td>
<td>0.001</td>
</tr>
</tbody>
</table>

**DISCUSSION** UC is a chronic inflammatory disease. Increasing attention has been recently given to the role of ROS in the pathogenesis of UC, because an inflamed intestine is exposed to oxidative stress generated by infiltrating macrophages and neutrophils within the lamina propria.

On the other hand, it is well known that an increased cancer risk occurs in tissues undergoing chronic inflammation. Many cancers develop at the sites of infection, chronic irritation, and inflammation.

The colon is more sensitive to oxidative damage because of the relatively low amount of antioxidants available in the mucosa. The high levels of DNA damage in IBD could represent a significant source of genomic instability. The accumulation of ROS could cause damage to specific genes involved in cell growth or differentiation or could cause changes in antioxidant enzyme levels.

Oxidative stress has been well documented in UC with increased ROS levels and decreased antioxidant levels in the inflamed mucosa, which ultimately contribute to chronic tissue damage. Increased ROS in the cell may lead to severe damage to macromolecules, for example DNA, causing base changes, strand breaks, and the altered expression of oncogenes. Increased oxidative stress and lipid peroxidation causing DNA damage and disturbance of cell signalling pathways, are implicated in human cancers, neurodegenerative diseases, and aging processes.

Oxidative damage to DNA is particularly important because there is growing recognition that such damage can initiate and promote carcinogenesis. During the last decade, human peripheral mononuclear leukocytes have been widely used to monitor environmentally induced genetic damage by a variety of methods, such as micronucleus, chromosome aberration, and sister-chromatid exchange assays. One of the useful methods of quantifying DNA damage is the single cell gel electrophoresis (comet) assay. Because of its simplicity and sensitivity, the comet assay has quickly gained acceptance as a genotoxicity assay. It has been reported that strand breaks arise from DNA damage generated by oxidative stress. We therefore used this method to measure DNA damage in circulating mononuclear leukocytes.

The comet assay is a well-established genotoxicity test that is easy to perform. It is quick, simple, and the observations are made at the level of single cells. It is an extremely useful tool that is used to assess the extent of endogenous DNA damage. The comet assay can potentially estimate DNA damage at the level of single cells and provide information on DNA damage in individual cells.

To our knowledge, there have been no data concerning the oxidative stress and DNA damage level in peripheral blood lymphocytes measured using the comet assay in patients with UC, particularly in relation to oxidative stress. In the present study, we for the first time observed that UC patients have increased peripheral DNA damage levels, TOS levels, and OSI values along with decreased TAC levels. Furthermore, when patients with UC were divided according to the disease activity, no differences were observed between active UC and inactive UC with respect to peripheral lymphocyte DNA damage, TOS, OSI, and TAC. Moreover, we found that peripheral lymphocyte DNA damage was significantly correlated with CRP, ESR, albumin levels, as well as platelet and leukocyte counts in patients with UC. Finally, these findings suggest that increased peripheral DNA damage may be related to activated phagocytic leukocytes generating excess amounts of ROS with subsequent increase in oxidative stress.

Previous studies have shown that DNA damage is involved in the pathophysiology of UC but the results are controversial. Lih-Brody et al. measured 8-OHdG concentration in the intestinal mucosa of the samples with UC. They reported no significant differences in the levels of 8-OHdG between UC samples and controls. On the other hand, D’Incà et al. investigated the role of free radical-mediated oxidative DNA damage during inflammation determined in patients with UC by measuring 8-OHdG. They observed that mucosal 8-OHdG concentrations were significantly increased in patients with both UC and dysplasia.
Antioxidant defense mechanisms are decreased in UC, presumably due to excessive inflammation. Recent studies have shown that the mucosa of patients with active UC has lower content of superoxide dismutase. Beno et al. observed a significant increase in GSH-Px activity in inflamed mucosa in a study on patients with active and inactive UC. However, Durak et al. reported no significant difference in the levels of tissue GSH-Px between patients with UC and controls. They observed that tissue malonyldialdehyde (MDA) levels in the patient group were significantly lower indicating that there was no oxidative stress in the mucosa and that defense mechanism was not impaired. In line with the study by Bhaskar et al., plasma MDA levels were not different between the patient and control groups. On the other hand, Bhaskar et al. observed that mucosal activities of antioxidant enzymes were not different between patients with UC and the control group. They reported no significant differences in the MDA levels between UC samples and controls.

Conventional drugs for treating UC in humans, such as 5-aminosalicylate (5-ASA), are potent ROS scavengers. Recent studies have shown that treatment of UC patients with 5-ASA compounds can prevent the development of carcinoma.

Our results indicate that increased peripheral DNA damage and oxidative stress is associated with decreased antioxidant levels and thus may be one of the pathophysiological mechanisms in the development of UC-associated colorectal cancer. Further studies on a larger group of patients are needed to confirm the mechanisms underlying the association of increased peripheral DNA damage and decreased antioxidant levels with the development of colorectal cancer in UC patients.

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REFERENCES


Uszkodzenie DNA limfocytów obwodowych i stres oksydacyjny u pacjentów z wrzodziejącym zapaleniem jelita grubego

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SŁOWA KLUCZOWE
- całkowity potencjał antyoksydacyjny
- całkowity status oksydacyjny
- elektroforeza rakietkowa pojedynczych komórek, uszkodzenie DNA w limfocytach obwodowych, wrzodziejące zapalenie jelita grubego

STRESZCZENIE

Wprowadzenie: Wroździejące zapalenie jelita grubego (ulcerative colitis – UC) to dość często występująca przewlekła choroba zapalna. Przewlekły proces zapalny może się przyczyniać do zwiększenia ryzyka choroby nowotworowej jelit poprzez akumulację produktów wynikających z uszkodzeń DNA. Wykazano już, że uszkodzanie DNA i stres oksydacyjny odgrywają istotną rolę w rozwoju UC, niemniej wyniki badań nadal pozostają niejednoznaczne.

CELE: Celem obecnego badania była ocena uszkodzeń DNA na obwodzie oraz stresu oksydacyjnego u pacjentów z UC.

PACJENTI I METODY: Do badania zakwalifikowano 20 pacjentów z UC oraz 20 osób stanowiących grupę kontrolną. Uszkodzenie DNA limfocytów obwodowych badano za pomocą elektroforeszy rakietkowej pojedynczych komórek (comet assay). Obliczono całkowity potencjał antyoksydacyjny osocha (total antioxidant capacity – TAC), całkowity status oksydacyjny (total oxidant status – TOS) oraz wskaźnik stresu oksydacyjnego (oxidative stress index – OSI).

WYNIKI: Poziom uszkodzenia DNA oraz TOS i OSI były znamienicie większe u pacjentów z UC niż w grupie kontrolnej (P < 0,001 dla wszystkich parametrów), podczas gdy wartości TAC były istotnie mniejsze (P < 0,001). Stwierdzono istotną statystycznie korelację pomiędzy uszkodzeniem DNA i wartościami TOS, TAC i OSI (odpowiednio r = 0,604, P < 0,001; r = −0,593, P < 0,001; r = 0,716, P < 0,001), jak również pomiędzy poziomem TAC a TOS i OSI (odpowiednio, r = 0,604, P < 0,001; r = −0,399, P < 0,05; r = −0,513, P < 0,05).

WNIOSKI: Nasze wyniki sugerują, że u pacjentów z UC zwiększenie uszkodzeń DNA na obwodzie oraz stresu oksydacyjnego wydają się związane ze zmniejszonym poziomem antyoksydantów, co może częściowo przyczynić się do rozwoju raka jelita grubego związanego z UC.