The Study of Oral Findings, Oxidative Stress and Antioxidant Vitamin E in Serum and Saliva of Crohn's Patients on Azathioprine Monotherapy and those on Combination of Anti-TNF-α Plus Azathioprine

Zainab Kh. Abbas, B.D.S. (1)  
Taghreed F. Zaidan B.D.S, M.Sc., Ph.D. (2)

ABSTRACT

Background: Crohn's disease (CD) is an immunological disorder associated with chronic inflammatory process of several unspecific regions of gastrointestinal tract but frequently detected in the terminal Ilium and proximal colon or both. This disease frequently presented with various oral manifestations as a consequence of inflammatory process of the disease, nutritional deficiency or medications side effects. Several therapeutic approaches have been developed for CD management that are targeting the inflammatory process and directed at controlling the host immune response. Immunosuppressants such as Azathioprine and anti-TNF α agents as well as the combination of them have been widely used as an effective therapeutic modality with a better chance of achieving clinical remission and decrease the risk of future complications.

Subjects, Materials, and Methods: Seventy-five subjects were incorporated in this study; fifty subjects presented with moderate to severe CD that were subdivided into two subgroups treated with different therapeutic modalities: Azathioprine monotherapy and Combination of anti-TNF-α biological agent (Infliximab) plus Azathioprine, while other twenty-five were age and gender matched healthy control subjects. Body mass index (BMI) of all participants was determined by weight (kg)/high (m²). Oral cavity of each patient was examined for the presence of oral manifestations. Blood and saliva samples were gained from each subject enrolled in this study and salivary flow rate (ml/min) was measured for those participants, then those samples were centrifuged and the supernatants frizzed for subsequent oxidative stress and antioxidants biomarkers assessment.

Results: The means of BMI and salivary flow rate were significantly higher in combination treated group than Azathioprine treated group. Fungal infection was the main oral findings that observed among Crohn’s patients on combination therapy compared with those on Azathioprine monotherapy. Assessment of oxidative stress and antioxidant activity revealed that the means of serum and saliva Malondialdehyde (MDA) were highly significantly higher in Azathioprine treated group than combination treated group, while Vitamin E (VE) level showed significant reduction in Azathioprine Crohn’s group as compared to combination treated group. Significant negative linear correlation was found between MDA level in serum and saliva with duration of treatment, while highly significant positive correlation was detected between VE levels and duration of treatment.

Conclusions: Oxidative stress was increased with reduced antioxidant level in both groups of Crohn’s patients. Treatment with Combination therapy approved to be effective in controlling oxidative tissue damage and enhanced antioxidants system in Crohn’s patients better than Azathioprine monotherapy. Fungal infection was the most predominant oral manifestations among Crohn’s patients on combination therapy as a result of concomitant immunosuppressive effect that accounted for reduced immune response to opportunistic infections.

Keywords: Crohn’s disease, Azathioprine, Combination therapy, Oxidative stress, Antioxidants. (J Bagh Coll Dentistry 2018; 30(1): 39-45).

INTRODUCTION

Crohn's disease is a debilitating inflammatory bowel illness characterized by granulomatous inflammation of the bowel wall. The inflammatory process is not limited to one region but can affect any part of the alimentary tract (1). This disease can be classified according to the age at the diagnosis, behavior, and location of the disease (Montreal classification) into A1, A2, A3 for ages that less than 16, from 17 to 40 and over 40 years respectively, and into B1, B2, B3 and P for disease behaviors that are representing non-structuring and non-penetrating, structuring, penetrating and perianal disease respectively and finally according to the location of the disease into L1, L2, L3 and L4 for ileal, colon, ileocolonic and isolated upper digestive sites (2).

Crohn's disease mostly affects males as compared to females at about 3:1 incidence ratio in Japan and Korea with the peak prevalence rate at the fourth decade of life (1). The specific etiological factor of this inflammatory disease is unclear and mostly known as a multifactorial disease, however, dysregulated intestinal immune balance is the main responsible factor of disrupting inflammatory response which represents the main source of reactive oxygen species (ROS) generation that approved to be the underlying pathological mechanism of CD (3). Crohn's disease is clinically diagnosed by the interaction of objective data obtained from endoscopy and histological examination with clinical signs and symptoms reported by patients (2). Chronic diarrhea, Abdominal pain, blood and/or mucus in stool and weight loss are represented the most common intestinal manifestations experienced by patients with CD (1).
Extraintestinal manifestations also detected in CD and oral cavity is frequently involved with these manifestations, the most specific oral manifestations that commonly associated with CD are lip swelling, deep linear ulceration, cobblestoning of buccal mucosa and tissue tags, other less specific manifestations such as angular cheilitis, candidiasis, oral aphthous ulceration, glossitis, and xerosomia might also be detected in the oral cavity of Crohn's patients (4). Changing of the patients lifestyle is the first step in the management of CD besides many pharmacological agents that are dependent in treatment of this disease with two common therapeutic approaches either by initiation with less intensive medications (e.g. corticosteroids or mesalazin) for controlling the symptoms and inducing remission, then followed by more intensifying pharmacological agents (e.g. anti TNF –α biological agents and immunosuppressant agents) in order to maintain remission (1), or by early depending of aggressive treatment as this approach is frequently associated with better clinical outcome and less further complications (5). An increase of infiltrated neutrophils and pro-inflammatory cytokines into the intestinal mucosa of Crohn's patients causing abundant amount production of free radicals and reactive oxygen species (ROS) which are unstable oxygen derivatives with potentially damaging effect (3). The main target of free radicals oxidizing effect is polyunsaturated fatty acids that presented in the cell membrane producing a chain of peroxidation reaction, a process causing membrane damage and various byproducts generation such as MDA (6). The harmful damaging effect of free radicals can be dampened by endogenous enzymes and dietary substances termed as antioxidants (7). These antioxidants have several detoxifying mechanisms in fighting off free radicals either by inhibiting, scavenging or repairing the oxidized biomolecules (8). Overcoming the biological antioxidant system by oxidants overproduction and the inability of this system to maintain the redox state of the body creates what is known, oxidative stress condition which is thought to be implicated in the pathogenesis of many pathological conditions (9). Increased ROS generation faced by reduction of enzymatic intestinal and blood antioxidants levels was highly detected in case of CD (3).

**MATERIALS AND METHOD**

A total of 75 participants were incorporated in this research study. Fifty subjects of them were presented with already diagnosed moderate to severe CD with the age range 17-45 years that have been recruited from gastroenterology and hepatology teaching hospital in Baghdad medical city attended for taking their routine medical treatment and according to their treatment, these patients were subdivided into two subgroups: Twenty-five Crohn's patients on oral immunosuppressant therapy (Azathioprine) 50mg twice daily, and other twenty-five patients were on Combination therapy with intravenous infusion of anti-TNF-α (Infliximab) 5mg/kg every 8weeks plus oral immunosuppressant (Azathioprine) 50mg twice daily. Crohn's patients with another systemic disease such as diabetes mellitus and hypertension were excluded from this study and those with periodontitis also excluded due to its effect on the results of oxidative stress in saliva. The control group consisted of twenty-five healthy subjects that matching the case groups in age and gender. BMI was determined by weighting (kg) the subjects in both case and control groups and measuring their lengths (m) then calculated as

BMl=weight (kg) / height (m²).

Each subject participated in this study was examined intraorally by using a sterile dental mirror and probe under good artificial light to detect any redness, ulceration or nodularity of the oral mucosa and other oral alterations seems to be associated with CD then they asked to collect saliva for ten minutes after rinsing their mouths thoroughly with water and spitting the first collected saliva to discard the remaining water, after that saliva was collected by spitting in a plastic tube every minute. Then salivary flow rate was calculated immediately by dividing the volume of collected saliva (ml) by the time of collection (min). Saliva flow rate (ml/min)=volume of saliva /collection time. By using plastic disposable syringe with stainless steel needle, gauge 21, about 8 ml of blood was withdrawn from antecubital vein from all subjects enrolled in this study then the collected samples (blood and saliva) were centrifuged at 3000 rpm for ten minutes and the clear supernatants were divided and transferred into three plastic tubes for freezing at (-20 °C) until the date of biochemical assessments. Oxidative stress was detected in serum and saliva samples by using thiobarbituric acid reactive substances (TBARS) assay to measure the lipid peroxidation MDA as described by Buege and Aust (10). While the antioxidant capacity of Crohn's patients was assessed by measuring individual antioxidants such as V.E levels in serum and saliva of both Crohn's and control groups. The antioxidant V.E level was determined by using V.E ELISA Kit (Shanghai, China) according to

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laboratory work instructions stated by the manufacturer.

RESULTS

Age and Gender: Seventy-five subjects were included in this study. Fifty subjects were presented with CD and those divided into two subgroups, 25 Crohn's patients treated with Azathioprine only with mean age 29.52±9.06 years and they included 15 (60%) males and 10 (40%) females, while other 25 Crohn's patients treated with combination of anti TNF-α biological agent (Infliximab) and Azathioprine with mean age 31.8±9.21 years and they consisted of 18 (72%) males and 7 (28%) females. Control group involved 25 subjects were looked healthy and included 15 (60%) males and 10 (40%) females with mean age was 28.6±7.42 years. Statistical analysis using F-test showed no significant difference (P>0.05) in age between study groups (Table 1).

Duration of treatment:
According to treatment, Crohn's patients in this study were subdivided into Azathioprine group and Combination therapy group. The patients in each group were classified according to the duration of treatment as showed in table 2. The results showed there was no significant difference (P>0.05) between the mean of duration on Azathioprine monotherapy (15.08±11.96 months) and that on Combination therapy (14.8±10.64 months).

Body mass index:
ANOVA showed there was a significant difference (P<0.05) among the mean of BMI of the study groups (Table 3). Continuing analysis with Tukey’s HSD test showed that the mean of BMI of the patients on Azathioprine was decreased significantly (P<0.05) than that in patients on Combination therapy and control group, while no significant difference (P>0.05) was shown between patients group on Combination therapy and control subjects (Table 4).

Salivary flow rate:
The results showed there was highly significant difference among the mean of salivary flow rate of the study groups (P<0.001) (Table 3). According to therapeutic options, the lowest mean of salivary flow rate was seen in patients on Azathioprine monotherapy that significantly differed (P<0.05) than that in patients on Combination therapy and highly significant (P<0.001) than control group as shown in table 4.

Oral Findings:
Intraoral examination of Crohn's patients showed that fungal infections (candidiasis and angular cheilitis) were the most prevalent oral findings among those on Combination therapy as it presented in 60% of them more than those on Azathioprine monotherapy group as it presented in 36% of them. Aphthous ulceration was the second predominant oral findings that seen among Crohn's patients and appear to be higher in the group on Azathioprine monotherapy (36%) as compared with those on Combination therapy (16%). Other oral findings like dysphagia, hairy tongue, atrophic glossitis, lip swelling and desquamative gingivitis were also reported in Crohn's patients in Azathioprine group and appear to be higher than that in Combination therapy group. While oral lichen planus was higher in patients on Combination therapy as illustrated in figure 1.

Table 1: Age and gender distribution of the study groups.

<table>
<thead>
<tr>
<th>Gender</th>
<th>Crohn's patients</th>
<th>Control</th>
<th>p-value</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aza.</td>
<td>Comb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>15</td>
<td>18</td>
<td>15</td>
<td>0.069</td>
</tr>
<tr>
<td>Female</td>
<td>10</td>
<td>7</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Mean ±S.D.</td>
<td>29.5±9.06</td>
<td>31.8±9.21</td>
<td>28.6±7.42</td>
<td>0.549**</td>
</tr>
</tbody>
</table>

*non-significant P>0.05 by using Chi-square
**non-significant P>0.05 by using ANOVA test

Table 2: Patients' distribution according to duration of treatment in Azathioprine and Combination Crohn's groups.

<table>
<thead>
<tr>
<th>Duration of treatment (months)</th>
<th>Azathioprine</th>
<th>Combination</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>%</td>
<td>No.</td>
</tr>
<tr>
<td>&lt; 12 months</td>
<td>11</td>
<td>44%</td>
</tr>
<tr>
<td>12 – 24 months</td>
<td>10</td>
<td>40%</td>
</tr>
<tr>
<td>&gt; 24 months</td>
<td>4</td>
<td>16%</td>
</tr>
</tbody>
</table>

Malondialdehyde (MDA) oxidative stress biomarker
Oxidative stress was determined in serum and saliva of Crohn's subgroups and control group as a lipid peroxidation byproduct MDA that showed highly significant increasing (P<0.001) among patients on Azathioprine monotherapy comparing with that in other study groups as shown in table 3. Analysis with Tukey's HSD test revealed that serum and saliva MDA levels of Crohn's patients on Azathioprine monotherapy were highly significantly increased (P<0.001) than that of patients on Combination therapy and control group. The same was shown between Crohn's...
patients on Combination therapy and control group (Table 4).

**Vitamin E (V.E) antioxidants biomarker:**
The comparison among the three study groups by ANOVA test revealed that there was highly significant reduction (p<0.001) in serum and saliva levels of V.E in Azathioprine treated group as compared with other study groups (Table 3). The results of Tukey’s HSD test showed that Azathioprine treated group showed a significant reduction (p<0.05) of serum and saliva V.E levels than that of Combination treated group and control group. The levels of serum and saliva V.E were significantly decreased (p<0.05) in patients on Combination therapy than control subjects (Table 4).

**The correlations between duration of treatment and oxidant, antioxidant parameters:**
The correlation between duration of Combination therapy and oxidative stress biomarker (MDA) showed highly significant, negative linear correlation, while the correlations with V.E levels were highly significant positive linear correlation. On the other hand, duration on Azathioprine monotherapy showed significant positive correlation with serum V.E, while no significant correlation was detected between serum and saliva MDA and saliva V.E.

Table 3: Means and S.D. values of all study parameters in the study groups.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control group No.=25</th>
<th>Azathioprine group No.=25</th>
<th>Combination group No.=25</th>
<th>P-value *</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI (Kg/m²)</td>
<td>25.46±2.59</td>
<td>23.73±2.09</td>
<td>25.20±2.18</td>
<td>0.02</td>
</tr>
<tr>
<td>Salivary flow rate (ml/min)</td>
<td>0.40±0.08</td>
<td>0.25±0.08</td>
<td>0.30±0.06</td>
<td>0.000</td>
</tr>
<tr>
<td>Serum MDA(µmol/L)</td>
<td>0.16±0.02</td>
<td>0.31±0.04</td>
<td>0.23±0.06</td>
<td>0.000</td>
</tr>
<tr>
<td>Saliva MDA(µmol/L)</td>
<td>0.12±0.03</td>
<td>0.24±0.06</td>
<td>0.19±0.08</td>
<td>0.000</td>
</tr>
<tr>
<td>Serum V.E(µmol/L)</td>
<td>3.17±0.95</td>
<td>1.85±0.92</td>
<td>2.44±0.87</td>
<td>0.000</td>
</tr>
<tr>
<td>Saliva V.E(µmol/L)</td>
<td>2.40±0.81</td>
<td>1.47±0.8</td>
<td>1.96±0.61</td>
<td>0.000</td>
</tr>
</tbody>
</table>

* Significant p>0.05 by using ANOVA test

Figure 3: Comparison of oral findings between Azathioprine monotherapy and Combination therapy Crohn's groups

Table 4: Multiple comparisons (p value) between study parameters in the study groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Groups</th>
<th>BMI</th>
<th>Flow rate</th>
<th>Serum MDA</th>
<th>Saliva MDA</th>
<th>Serum V.E</th>
<th>Saliva V.E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azathioprine</td>
<td>Combination</td>
<td>0.037*</td>
<td>0.020*</td>
<td>0.000*</td>
<td>0.020*</td>
<td>0.001*</td>
<td>0.018*</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>0.025*</td>
<td>0.000*</td>
<td>0.000*</td>
<td>0.000*</td>
<td>0.017*</td>
<td>0.020*</td>
</tr>
<tr>
<td>Combination</td>
<td>Control</td>
<td>0.916</td>
<td>0.000*</td>
<td>0.000*</td>
<td>0.000*</td>
<td>0.001*</td>
<td>0.022*</td>
</tr>
</tbody>
</table>

BMI: Body mass index, MDA: Malondialdehyde, V.E: Vitamin E
* Significant p>0.05 by using Tukey’s HSD test
DISCUSSION

Age and Gender: The mean age of Crohn's patients on Azathioprine monotherapy was 29.52 years, while the mean age of those on Combination therapy was 31.8 years. The importance of age in CD prediction was stated by Aljebreen et al. who found the mean age at the diagnosis of this disease was 25 years with peak occurrence rate at the ages between 15-30 years. With respect to gender distribution according to treatment modalities, CD in both Azathioprine and Combination treated groups showed marked male predominance on the account of female and this agreed with findings of other studies.

Body mass index: According to results of the present study patients on Combination therapy presented with significantly higher (p<0.05) body mass index than those on Azathioprine monotherapy, while no significant difference (p>0.05) was found as compared with control group. This may be due to the effective role of Combination therapy in induction of clinical remission and mucosal healing achievement; therefore, improved nutritional status causing an increase of BMI as mentioned by Kang et al. who reported that pediatric and adolescent patients with moderate to severe luminal CD treated early with Combination therapy showed a significant increase in mucosal healing and BMI.

Salivary flow rate: The results of this study found that salivary flow rate of Crohn's subgroups was highly significantly reduced (P<0.001) than that in control group. Crohn's disease is an autoimmune inflammatory condition associated with a granulomatous reaction that may involve salivary glands and causing dysfunctional changes of these glands. This may be the underlying cause of decreased salivary secretion in these patients. In term of salivary flow rate according to treatment type of CD, the results found that the mean of salivary flow rate in patients on Combination therapy was significantly higher (P<0.05) than patients on Azathioprine monotherapy.

An in vitro study performed on human salivary gland (HSG) cells suggested that TNF-α play a critical role in extrinsic apoptotic pathway induction and activation of intercellular adhesion molecule-1 resulting sticky apoptotic bodies within these cells and this accounted for morphological and functional disruption of salivary glands. So the difference in salivary flow rate between Azathioprine and Combination therapy treated groups may be a result of an improved response to TNF-α targeting effect of Infliximab.

Oral findings: Fungal infections including candidiasis and angular cheilitis were seen in Crohn's patients on Combination therapy more than in those on Azathioprine monotherapy and this attributed to the extra immunocompromising effect of concomitant immunosuppressive agents usage that leads to reduce the resistance and increased incidence of opportunistic infections. According to findings of this study lip swelling was found in 8% of Crohn's patients on Azathioprine monotherapy, while no patient on Combination therapy presented with this complication and this in line with Hoekman et al. who reported a case of CD manifested with lip swelling that showed a resistance to Azathioprine monotherapy before the initiation of Combination therapy which succeed in achieving clinical response and reduce the severity of lip swelling and this may be attributed to incomplete controlling of the underlying intestinal granulomatous inflammation of CD by immunosuppressive monotherapy. The results of this study also found that 4% of the patients on Combination therapy presented with oral lichen planus. The association between lichen planus and CD has been interpreted as CD could be associated with other immunological conditions including lichen planus. Increase occurrence among patients on Combination therapy may be attributed to the side effect of anti-TNF-α therapy as mentioned by Andrade et al. who reported the presence of oral lichen planus in females Crohn's patients after treating with TNF-α inhibitors.

Oxidative stress marker (MDA): It has been found that Crohn's patients in Azathioprine and Combination treated groups were showed highly significant increasing (p<0.001) of serum and saliva MDA than control subjects. Elevated MDA level in CD may be associated with the inflammatory characteristics of the disease. An intimate relation between free radical metabolism and the inflammatory process has been reported by Yamada and Grisham; Kruidenier et al.; Alzoghabi. Serum and saliva MDA levels were lower in patients on Combination therapy than in those on Azathioprine monotherapy and this reduction attributed to additive effect exerted by both medications in controlling the intestinal inflammation, the main source of ROS production.

Vitamin E antioxidant: The results revealed that V.E levels were highly significantly lowered (p<0.001) in Crohn's treated groups than that of the control group. V.E acts as a lipid peroxidation inhibitor and has free radicals scavenging ability, therefore; excessive free radicals generation in Crohn's patients attributing V.E depletion in those patients. Impaired intestinal absorption of dietary vitamins in Crohn's patients was also thought to be highly implicated in reduced V.E level. Reduced level of...
MDA and better intestinal vitamins absorption in patients on Combination therapy may be accounted for increased V.E level than those on Azathioprine monotherapy.

The correlations between duration of treatment and oxidant, antioxidant biomarkers

The results revealed that increasing duration of Combination therapy was associated with gradual MDA reduction and improved antioxidants level in serum and saliva of Crohn’s patients on this therapeutic approach and this probably related to amplified anti-inflammatory and immune-suppressive effect of both immunomodulator agents (Infliximab and Azathioprine) in maintaining reduction of oxidative stress by interfering with the source of inflammation, mainly pro-inflammatory by TNF-α cytokine and circulating inflammatory cells.

CONCLUSION

High level of oxidative stress was detected in Crohn’s patients, suggested that oxidative stress play a critical role in the pathogenesis of CD. Clear significant reduction of antioxidant capacity in Crohn’s patients was indicated by significant lowering of serum and saliva V.E antioxidant level more than that in control group. However, patients on Azathioprine monotherapy showed significant antioxidants reduction by comparing with those on Combination therapy, this reduction pointed out excessive antioxidants consumption in fighting off free radicals overproduction by tissues with CD. Reduced salivary flow rate among patients with CD, as well as, impaired immune response accounted for increase opportunistic infections in those patients.

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The study

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The study

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