17) IMPACT OF THE LEVEL OF THE INTESTINAL SHORT CHAIN FATTY ACIDS IN INFLAMMATORY BOWEL DISEASE PATIENTS VERSUS HEALTHY SUBJECTS

Authors:

1. Dr. Asutosh. P. Chauhan (MD Biochemistry), Assistant professor, Biochemistry, Medical College and SSG Hospital, Vadodara, Gujarat. 
   Email- dr_asutoshchauhan@yahoo.com
2. Dr. Ashish. Madiya (MD Pathology), Tutor, pathology, Medical college and SSG Hospital, Vadodara, Gujarat
3. Dr. Prakash Bhabhor (MD Pharmacology), Associate Professor, GMERS Medical College and Hospital Gotri, Vadodara, Gujarat.

Abstract:
The aim of this study was to determine the changes of short chain fatty acids (SCFAs) in feces of inflammatory bowel disease (IBD) patients compared to healthy subjects. SCFAs such as pyruvic, lactic, formic, acetic, propionic, isobutyric and butyric acids were analyzed by using high performance liquid chromatography (HPLC). This study showed that the level of acetic, 162.0 µmol/g wet feces, butyric, 86.9 µmol/g wet feces, and propionic acids, 65.6 µmol/g wet feces, decreased remarkably in IBD fecal samples when compared with that of healthy individuals, 209.7, 176.0, and 93.3 µmol/g wet feces respectively. On the contrary, lactic and pyruvic acids showed higher levels in fecal samples of IBD than in healthy subjects. In the context of butyric acid level, this study also found that the molar ratio of butyric acid was higher than propionic acid in both fecal samples. This might be due to the high intake of starch from rice among Malaysian population. It was concluded that the level of SCFAs differ remarkably between fecal samples in healthy subjects and that in IBD patients providing evidence that SCFAs more likely play an important role in the pathogenesis of IBD.

Keywords: Organic acid, human fecal, IBD, HPLC.

INTRODUCTION
Short chain fatty acids (SCFAs), are carboxylic acids with 1 to 6 carbon atoms that include different other functional groups, such as hydroxyl or dicarboxyl. In human, SCFAs arise from bacterial fermentation of carbohydrates, proteins, peptides and glycoprotein precursors (1, 2). SCFAs such as acetic, propionic and butyric acids are mainly formed during microbial fermentation of carbohydrate in the colon. The most important role of SCFAs in colonic physiology is their trophic effect on the intestinal epithelium. In human, SCFAs production from inulin-type fructan can increase the metabolic activity, pointing to trophic effects for colonocytes. Approximately 80-90% of SCFAs, which are produced from the breakdown of dietary food, are absorbed in colon while the rest are excreted in feces. SCFAs content in feces could be used as a biomarker for the physiological processes in the organisms as well as for the effect of nutritional interventions (1). The level of SCFA content in fecal samples have been
shown to be related with some diseases such as IBD, irritable bowel syndrome (IBS), cardiovascular disease (CVD), diarrhoea (3), and cancer. It was also reported that increased lactic acid may modulate diarrhea in UC. In addition, fecal SCFAs, acetic and propionic acids, in patients with diarrhea-dominant IBS were found to be of lower levels than in healthy individuals (4, 5). Therefore, there has been increasing evidence that the majority of SCFAs play an essential role in maintaining the health of colonic mucosa. However, butyric, acetic, and propionic acids have mainly been emphasized. In addition, butyric acid appears to induce differentiation of tumor cell lines (6). Several methods were used to analyze the fecal SCFAs in rat and human samples. For example, a rapid and reliable gas chromatographic (GC) method has been developed to determine eight SCFAs, in the colonic and fecal samples of rats and humans. In addition, methods such as vacuum ultrafiltration followed by GC, ion chromatography (IC), and IC with solid phase extraction were used for the determination of SCFAs in fecal samples. In addition to GC, high performance liquid chromatography (HPLC) has also been applied to the analysis of fecal SCFAs in humans (7). HPLC is convenient for the quantification of SCFAs, and it is less time-consuming. To date, data on IBD cases is still scarce in that very few studies, if any, were conducted on the content and the role of SCFAs in feces of healthy and IBD subjects. Therefore, the current study was conducted to determine the level of fecal SCFAs (pyruvic, lactic, acetic, formic, propionic, butyric and isobutyric acids) in IBD patients by using HPLC methods. The level of each fecal SCFA was compared to counterpart samples from healthy individuals.

MATERIALS AND METHODOLOGY

Chemicals and Reagents

Formic acid, 98-100%, and acetic acid, 100%, were obtained from Merck, pyruvic acid, 99%, and propionic acid, 99%, were obtained from Merck, isobutyric acid, 99%, was obtained from Sigma, lactic acid, 100%, and butyric acid, 99.5%.

The Study Population and Fecal Samples

Fecal samples were obtained from 50 healthy subjects (male = 18, female = 32) and 8 IBD (male = 6, female = 2) subjects from March 2010 to December 2011 in vadodara, Gujarat. The age of the studied patients ranged from 34 to 68 years and the age for the healthy subjects ranged from 22 to 55 years. Six of IBD patients were at remission phase except for 2 UC were at active phase; nevertheless, all of the involved patients showed no diarrheal symptoms within 2 weeks before the time of sampling. The nutritional status of the involved patients was within average and patients did not change their nutritional habit during sampling. The IBD samples were collected from patients who had been diagnosed as CD (n=2) and UC (n=6). Diagnosis of IBD was confirmed in all cases by colonoscopy and histology. The main symptoms of the involved IBD patients within the last month were: for UC, loose stools (1 patient), low grade fewer (2 patients), mild diarrhea with blood stain (1 patient), abdominal pain (1 patient), and abdominal distension (1 patient), for CD, patients did not have remarkable symptoms. Furthermore, IBD patients did not experience any extra-intestinal manifestations. It is noteworthy to mention that IBD patients and the healthy volunteers did not receive antibiotics, probiotics, and prebiotics one month prior to the samples collection. Moreover, IBD patients did not receive mesalazine 2 weeks before sampling. The fecal samples were collected into clean sterile containers. They were immediately
taken to the laboratory and kept frozen at -20°C for analysis. Dealing with human subjects was carried out within the scope of the ethical principles of biomedical research. All of the involved subjects signed formal written consents.

**Preparation of Samples for Analysis**

Fecal samples of weight 0.2 g were used and diluted at ratio 1:4 to 1:8 (w/v) in sterile distilled water. The samples were then vortexed for 1 min and the homogenate was centrifuged at 10,000g for 10 min. The SCFA-containing supernatant was filtered through cellulose acetate membrane with a pore size of 0.2 µm and stored at -20°C until HPLC analysis.

**Determination of Organic Acids**

SCFAs analyses were carried out by using HPLC. Briefly, 40 µl of fecal samples extraction were injected directly into HPLC System. SCFA in fecal samples were separated using an ionic exchange resin, at 65°C. The target compounds were detected using a UV detector set at wavelength of 210 nm. Filtered 0.01 N H2SO4, through 0.45 µm nylon membrane, was used as a mobile phase at a flow rate of 0.6 ml/min.

**Preparation of Calibration Standard Curve**

Quantification of SCFAs in fecal samples was carried out using external calibration standard curves method. Seven calibration standards were prepared at six levels of concentration ranging from 0.005M to 0.03M for pyruvic acid, 0.01M to 0.06M for formic acid and acetic acid, and 0.02M to 0.12M for lactic acid, propionic acid, isobutyric acid and butyric acid. The reference samples were injected repeatedly for nine times to measure the retention time. The calibration curves were constructed by plotting the relative peak area versus the molarity of solution. Fecal SCFA concentrations were expressed as mean µmol per gram wet weight feces using the following equation as described by Hoshi et al. (8) with modification. Fecal SCFA (µmol/g) = [organic acid in fecal contents (mmol/ml) X Vd (ml) X 1000]/ Wet weight feces (g) Whereas: Vd = Total Volume of Dilution

**Statistical Analysis**

Data analysis was conducted. The normality of data was checked using Anderson-Darling test before statistical analysis was done. Differences between means of SCFA concentration between healthy and IBD groups were analyzed by using unpaired Student’s *t*-test. The means were considered statistically significant at *P*<0.05. Data was expressed in mean ± SEM (µmol/g wet faeces).

**RESULT**

**Validation of Retention Time for Analytical Methods**

The reference standard in different concentrations was analyzed on three different days to show the retention times. Since external standard was used for calibration, the method requires precise analytical technique and requires that the detector sensitivity must be constant from day to day if the calibration curve is to remain valid. Samples
were injected nine times in different concentrations and the average of retention time (Rt) was used. From the analysis, the Rt for pyruvic, lactic, formic, acetic, propionic, isobutyric and butyric acids was 9.28, 12.07, 13.38, 14.60, 17.28, 19.49, and 21.82 min respectively.

Short Chain Fatty Acids in Fecal Samples

The mean concentrations of SCFA in fecal samples of healthy and IBD subjects are shown in Table 1. From the table, results revealed that the mean concentrations of butyric and propionic acids, 86.9 and 65.6 µmol/g wet feces, respectively in IBD fecal samples were lower than in healthy subjects, 176.0 and 93.3 µmol/g wet feces respectively (P<0.05). Acetic acid was also lower in fecal samples of IBD patients than of healthy subjects but the difference was not so significant (p=0.16). The fecal concentration of formic acid, isobutyric, lactic and pyruvic acids were also determined and compared between healthy and IBD groups. Formic acid was only detected in five healthy subjects and isobutyric acid was only detected in two samples of healthy group while no detection of formic and isobutyric acids was found in the fecal samples of IBD patients. In contrary, the fecal concentration of lactic and pyruvic acids was lower in healthy subjects, 24.5 µmol/g and 0.5 µmol/g respectively, than in IBD patients, 73.5 µmol/g and 2.1 µmol/g respectively, but this difference was not significant (P >0.05).

Molar Ratio of Main SCFA in Fecal Samples

There has been no report about the molar ratio of main SCFAs in fecal samples in Indian subjects. Therefore, the molar ratio of acetic, propionic and butyric acids were calculated. From the current study, the molar ratio of acetic: propionic: butyric acids in fecal samples of healthy subjects were 45:20:38 and in fecal samples of IBD were 49:20:27. This finding showed that the molar ratio of butyric acid and propionic was 1.5 and 1.35 in healthy and IBD subjects, respectively.

DISCUSSION

In this study, it was shown that butyric and propionic acids were decreased significantly in IBD subjects. This finding was similar to that reported by Takaishi et al. (7) who revealed that the concentrations of butyric and propionic acids were significantly decreased in IBD patients than in healthy controls. In addition, Vernia et al. (11) reported similar decreasing trend of butyric acid in UC patients. The decreasing level of butyric acid could be due to the assumption that the distribution of intestinal microbiota changes in IBD patients (7). Furthermore, it was reported that the fecal proportion of butyric acid in patients with UC increased after consuming oat bran. Moreover, it was suggested that when the butyric acid is low in UC patients, the risk of colon cancer increases (9). On the other hand, the increasing propionic acid was shown to be associated with decreasing serum cholesterol in blood (10). Comparing acetic acid concentration between healthy and IBD fecal samples, Takaishi et al. also reported that the acetic acid in IBD feces was not significantly lower than in healthy control and they found that the concentration of acetic acid in CD was lower than in UC (7). Nilsson et al. reported that the mean level of acetic acid in healthy subjects (n=20) before giving oat bran diet was 54.2 µmol/g wet feces ranging from 19.5 µmol/g to 126.2 µmol/g of feces.
They showed that, after 8 weeks of giving oat bran, the mean level of acetic acid increased 77.2 µmol/g wet feces.

Table 1. SCFA in Fecal Samples of IBD Patients and Healthy Subjects

<table>
<thead>
<tr>
<th></th>
<th>Healthy Subjects (n=50)</th>
<th>Range</th>
<th>%PC*</th>
<th>IBD Subjects (n=8)</th>
<th>Range</th>
<th>% PC</th>
<th>P-values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetic Acid</td>
<td>209.7±14.0</td>
<td>21.5 – 400.5</td>
<td>(100)</td>
<td>162.0 ± 28.0</td>
<td>64.2 – 308.9</td>
<td>(100)</td>
<td>0.160</td>
</tr>
<tr>
<td>Butyric Acid</td>
<td>176.0 ± 16.0*</td>
<td>45.2 – 503.3</td>
<td>(100)</td>
<td>86.9 ± 21.0*</td>
<td>32.7 – 204.2</td>
<td>(100)</td>
<td>0.004</td>
</tr>
<tr>
<td>Propionic Acid</td>
<td>93.3 ± 5.3*</td>
<td>33.8 – 185.1</td>
<td>(100)</td>
<td>65.6 ± 5.3*</td>
<td>45.1 – 91.7</td>
<td>(100)</td>
<td>0.001</td>
</tr>
<tr>
<td>Lactic Acid</td>
<td>24.5 ± 4.8</td>
<td>0 – 120.6</td>
<td>(48)</td>
<td>73.5 ± 37.0</td>
<td>0 – 254.4</td>
<td>(62.5)</td>
<td>0.237</td>
</tr>
<tr>
<td>Formic Acid</td>
<td>21.5 ± 9.7</td>
<td>0 – 364.2</td>
<td>(10)</td>
<td>No detection</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pyruvic Acid</td>
<td>0.5 ± 0.3</td>
<td>0 – 16.4</td>
<td>(8)</td>
<td>2.1 ± 1.3</td>
<td>0 – 10.1</td>
<td>(37.5)</td>
<td>0.261</td>
</tr>
<tr>
<td>Isobutyric Acid</td>
<td>17.9 ± 13.2</td>
<td>0 – 591.3</td>
<td>(4)</td>
<td>No detection</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*PC = Positive cases.

Results are expressed in µmol/g wet feces (mean ± SEM).

Value marked with asterisk are significantly different between two groups ($P<0.05$).

Ranging from 22.9 to 125.6 µmol/g of feces ($P<0.001$). And acetic acid absorption in the colon has been shown to increase cholesterol synthesis. However, addition of propionate to acetate resulted in no significant rise in cholesterol. With respect to lactic acid, the current study yielded similar results to a previous report stating that higher lactic acid concentration was found in both of fecal UC and CD samples than in healthy subjects (3). High concentration of lactic acid was associated with higher risk of diarrhoea and mucosal inflammation (2). The importance of low lactic acid concentration in fecal samples of healthy subjects is still unknown. However, lactic acid can be further metabolized by propionibacteria to propionic acid and acetic acid. In this study, fecal formic acid was only detected in five healthy subjects. Quantification of formic acid in fecal samples was always unsatisfactory and frequently at very low or undetectable level. This might be attributed to the fact that formic acid has been believed to be formed by microorganisms in the colon only at the initial phase of dietary fermentation. Furthermore, formic acid is an intermediate product, not an end-product, of bacterial fermentation and is converted readily to CO2 and water. Furthermore, formic acid was probably metabolised by bacterial enzymes during incubation at 50°C. In addition, it is extremely volatile and losses cannot be avoided during preparation of sample. For these reasons formic acid in feces is rarely detected. Takaishi et al. (7) and Vernia et al. (3) showed that pyruvic and succinic acids in fecal IBD were higher than in healthy subjects and they found that the concentration of pyruvic acid in patients with UC was significantly higher than in patients with CD. Their findings are similar to that of current study that pyruvic acid was higher in fecal IBD than in healthy group. However, this
increase was not significantly different. Other SCFAs such as iso-butryic, \( n \)-valeric, iso-valeric and \( n \)-caproic acids were presented in minor amounts in the human colon. All these SCFAs were too low to be detected. The branched chain fatty acids (BCFA), iso-butryic and iso-valeric acids are primarily produced from catabolism of protein particularly from branched amino acids fermentation. An increase of BCFA tends to be observed only when carbohydrate is limited. Approximately, these SCFAs account for 90 to 95% of total fatty acids. Of the SCFAs, the major products are acetic, propionic, and butyric acids which are commonly found in proportions approximately 60:20:20 (acetic: propionic: butyric) (1). However, these ratios are very rare to achieve in practice. The patterns of the molar ratio of SCFA awere tributed to the bacterial species present. In addition, it was also influenced by the composition of diet and type of indigestible carbohydrates. Moreover, the molar ratio of butyric and propionic acids was 1.5 and 1.35 in healthy and IBD subjects which might be due to the high intake of starch from rice. The possible anti-inflammatory mechanism of SCFAs, namely propionic, butyric, and acetic acids, are still not clarified adequately. However, a recent study showed that propionic and butyric acids were equipotent, whereas acetic acid was less effective, at suppressing NF-kappa B reporter activity, inflammation-related gene expression and cytokin release \textit{in vitro}. Therefore, these findings suggested that propionic and acetic acids, in addition to butyric acid, could be useful in the treatment of inflammatory disorders, including IBD (11). Moreover, it was shown that butyric acid suppresses nuclear factor-kappa B activation via GPR109A receptors in normal and cancer colon cell lines as well as in normal mouse colon. This study showed that GPR109A mediates the tumor-suppressive effects of the bacterial fermentation product, butyric acid in colon (12). In addition, it was confirmed that SCFA, adenine nucleotides, and phospholipids can modulate intestinal epithelial repair mechanisms.

**CONCLUSION**

From this study, it was concluded that the level of SCFAs might play an important role in the pathophysiology and/or progression of IBD. The current study showed that the level of SCFAs differs remarkably between the fecal samples of healthy subjects and these of IBD patients. As compared to the healthy subjects, acetic, butyric and propionic acids decreased dramatically in fecal samples of IBD as well as formic and isobutyric acids were not detectable in fecal samples of IBD while the level of acetic and pyruvic acids increased in feces of IBD patients. In this study, the molar ratio of butyric acid also showed higher proportion than propionic acid in fecal samples. However, future studies need larger sample size to determine more precisely the distribution of SCFAs among IBD patients in the region. The relationship between rice consumption and the reduction of IBD incidence also need to be determined since the incidence of IBD in this region is still not very common.
REFERENCES


