Effects of pH on the Deconjugation of Conjugated Bilirubin in Human Bile

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SHINYA, F., TOSHIMA, T., TAKAHASHI, W. and SUZUKI, N. Effects of pH on the Deconjugation of Conjugated Bilirubin in Human Bile. Tohoku J. exp. Med., 1985, 147 (3), 281–293 — The enzymatic activity of bacterial $\beta$-glucuronidase plays an essential role in the formation of calcium bilirubinate in bile. There are, however, many unsettled problems such as methodology of the assay for its enzymatic activity. In the present study (1) the azopigments from mono-conjugated bilirubin (MCB) and unconjugated bilirubin (UCB) in native bile were semiquantitatively determined, (2) the deconjugation of conjugated bilirubin (CB) in bile was estimated with azopigment analysis and (3) factors affecting the deconjugation of CB in bile, especially for pH value, were investigated. CB in bile was stable at physiologic pH during 6-hr incubation at 37°C, but was hydrolyzed at alkaline pH. At physiologic pH, addition of $\beta$-glucuronidase from $E. coli$ hydrolyzed CB in bile and increased MCB and UCB in bile. Based upon the results mentioned above, it is suggested that alkaline pH and enzymatic activity of $\beta$-glucuronidase should cause the increase of UCB in bile. It can be said that $\beta$-glucuronidase is essential for the formation of calcium bilirubinate gallstone at physiologic pH. ——— $\beta$-glucuronidase ; calcium bilirubinate gallstone ; diazo reaction ; hydrolysis of conjugated bilirubin ; unconjugated bilirubin

For the mechanism of the formation of calcium bilirubinate stone, the enzymatic activity of bacterial $\beta$-glucuronidase is presumed to play an essential role (Maki 1966). Bilirubin glucuronide in bile is hydrolyzed into unconjugated bilirubin (UCB) and glucuronic acid by $\beta$-glucuronidase, and calcium combines with the liberated UCB at its carboxyl radical, yielding water-insoluble calcium bilirubinate. It is necessary for the formation of calcium bilirubinate that the enzymatic activity of $\beta$-glucuronidase is increased by bacterial infection, and the inhibitory activity decreases. In fact, it has been reported that $\beta$-glucuronidase activity (pH 6.6–7.15) was found to be perceptibly increased in bile specimens which were sampled from patients with calcium bilirubinate stone (Maki et al. 1962). However $\beta$-glucuronidase activity has been determined by utilizing an ethereal glucuronide as substrate for assay and, the increase of UCB has not yet been directly demonstrated in bile from patients with calcium bilirubinate stone.
In the present study mono-conjugated bilirubin (MCB) and UCB in bile were semiquantitatively determined before and after incubation at various pH by use of direct diazotization of bilirubin in bile (Shinya 1984). Since di-conjugated bilirubin is hydrolyzed into UCB or MCB, azopigments from UCB and MCB are considered to be good indices of deconjugation of CB in bile. By analyzing these indices, the factors affecting the deconjugation of CB in bile were investigated.

**Materials and Methods**

*Bile samples.* Human hepatic bile was obtained through T-tube drainage from patients who had been operated at least before 10 days for cholelithiasis and acalculous cholecystitis, and through percutaneous transhepatic biliary drainage (PTBD) from patients with normalized serum bilirubin level who did not suffer from gallstone disease. All bile samples were prepared and stored on ice under the protection from light.

*Buffers and chemicals.* 2, 6-Di-tert.-butyl-p-cresol (Eastman Organic Chemicals, Rochester, N.Y., USA) was mixed with acetone-ethanol, 1:1 (v/v), at a concentration of 15 mg/ml and used as reaction-accelerating agent in diazotization by p-iodoaniline. Bacterial β-glucuronidase (from *E. coli*, Type IX) was obtained from Sigma Chemical Company (St. Louis, Mo, USA). 3', 4'-Dideoxykanamycin B (DKB) was obtained from Meiji Seika Kaisha, Ltd. (Tokyo). All other chemicals were obtained from Wako Pure Chemical Industries (Osaka). UCB was used without further purification.

Acetate buffer at 0.2 M (ionic strength \( \mu = 0.2 \)) was used for pH 5.0 and 5.5; 0.2 M phosphate buffer (\( \mu = 0.2 \)) for pH 6.0 to 7.5; and 0.2 M borate buffer (\( \mu = 0.7 \)) for pH 8.0 to 9.0.

**Determination of β-glucuronidase activity and total bilirubin concentration.** The method for quantitative determination of β-glucuronidase in bile was basically the same as that described by Fishman et al. (1948). The essential differences from their method were that p-nitrophenyl-β-D-glucuronide was employed as substrate instead of phenolphthalein mono-β-glucuronide (Konishi 1965) and that the samples were diluted with 0.2 M phosphate buffer (pH 6.8) because optimal pH for bacterial β-glucuronidase had ranged from 6.6 to 7.15 (Maki et al. 1962). Total bilirubin was determined by Malloy-Evelyn method (Malloy and Evelyn 1937).

**Quantitative analysis of azopigments.** CB and UCB in bile were diazotized by diazonium salts of ethyl anthranilate and p-iodoaniline respectively (Heirwegh et al. 1974). Bile samples were diluted by distilled water to approximately 10 mg/100 ml of total bilirubin concentration by distilled water just before diazotization. Azopigments were separated by thin layer chromatography on pre-coated silica gel plate (DC-Kieselgel F254, from Merck AG, Darmstadt, West Germany). They were developed at room temperature in the dark with chloroform-methanol-water (65:25:3, by volume) for 15 cm, and dried in the air stream. The separated azopigments were quantitatively determined by dual-wavelength TLC scanner (CS-900, Shimazu, Tokyo) as described by Uematsu et al. (1980). The plates used for TLC scanning were freshly developed. \( \alpha_{OE} \) and \( \alpha_{OF} \) values of each bile sample were calculated from the method as previously reported (Shinya 1984).

**Effects of pH on the deconjugation of CB in bile.** Bile samples obtained from 10 patients were used; 6 with calcium bilirubinate gallstone, 1 with mixed gallstone and 3 with malignant biliary tract disorder. One ml of bile sample was buffered in the range of pH 5.0 to 9.0 with 1.5 ml of various buffer solutions. Approximately 4 \( \mu g/ml \) of DKB was added to buffered bile samples to avoid the enzymatic activity induced from bacteria which increased during incubation. And the mixtures were incubated for 6 hours in brown-colored screw vials under argon at 37°C and with the protection from light. Total bilirubin concentration, and \( \alpha_{OE} \) and \( \alpha_{OF} \) values of these mixtures were determined before and after
Commercial UCB was dissolved in 0.1 N NaOH. This solution was buffered between pH 7.0 and 8.0 by various buffer solutions. Total bilirubin was determined before and after incubation under the same condition as previously described.

Effects of bacterial $\beta$-glucuronidase on the deconjugation of CB in bile. Bile samples from 9 patients were used; 5 with calcium bilirubinate gallstone, 1 with mixed gallstone, 1 with acalculous cholecystitis and 2 with malignant biliary tract disorder. Bacterial $\beta$-glucuronidase was dissolved in phosphate buffer (pH 7.5), and its enzymatic activity was adjusted between 20,000 and 2,500,000 Fishman units by twofolds dilution method. One ml of $\beta$-glucuronidase solution was added to the equal volume of bile sample and mixture was incubated for 6 hr under argon at 37°C with the protection from light. Before and after incubation the concentration of total bilirubin was determined. UCB and CB in the mixture were diazotized, and $\alpha_{OE}$ and $\alpha_{OF}$ values were calculated.

Statistical method. Results were expressed as mean ± s.e. Means of the calcium bilirubinate gallstone group and the control group were compared by Student's $t$-test.

RESULTS

Analysis of native bile samples

Bile from 15 patients with calcium bilirubinate gallstone disease and 11 controls were used in the present investigation (Table 1). Control group consisted of 3 patients with cholesterol gallstone disease, 1 with acalculous cholecystitis and 7 with malignant biliary tract disorder.

pH of the bile samples averaged 7.65±0.04, ranging from 7.18 to 8.00 and their total bilirubin was 46.36±5.01 mg/100 ml, ranging from 11.7 to 109.5 mg/100 ml. No statistically significant difference was found between the calcium bilirubinate stone group and the control group.

$\alpha_{OE}$ of the calcium bilirubinate stone group was slightly higher than that of the control, but no statistically significant difference was noted.

Effects of $3', 4'$-dideoxykanamycin B (DKB) on bacterial $\beta$-glucuronidase activity

DKB at concentration of 3.12 $\mu$g/ml demonstrates a strong antibacterial activity against 30 out of 36 strains of E. coli isolated from clinical materials (Nakazawa et al. 1974). The inhibitory effect of DKB on bacterial $\beta$-glucuronidase is shown in Fig. 1, but the effect was not perceptible at 4 $\mu$g/ml.

Incubation time

One ml of bile sample was mixed with 1.5 ml of buffer solution. The mixture was incubated under the same condition as those previously described. Figs. 2 and 3 show the percentages of $\alpha_{OE}^{-}$ and $\delta$-fractions of the mixtures against incubation time. At alkaline pH, an increase in $\alpha_{OE}$ and $\alpha_{OF}$ values and a decrease in $\delta$ values were observed within 2 hr. At neutral to acid pH, however, incubation for 6 hr was necessary in order to observe the changes in azopigment pattern.
Fig. 4 shows total bilirubin in bile sample against pH after 6-hr incubation. Total bilirubin concentration in both calcium bilirubinate stone group and control group was 80 to 90% of that before incubation at neutral pH (6.0-8.0). At acid pH (5.0-6.0), total bilirubin concentration in both groups apparently decreased.

**Table 1. Characteristics of bile samples**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Control group ( (n = 11) )</th>
<th>Calcium bilirubinate stone group ( (n = 15) )</th>
<th>Total ( (n = 26) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.72 ± 0.04</td>
<td>7.61 ± 0.06</td>
<td>7.65 ± 0.04</td>
</tr>
<tr>
<td>Total bilirubin (mg/100 ml)</td>
<td>43.45 ± 4.79</td>
<td>48.50 ± 7.89</td>
<td>46.36 ± 5.01</td>
</tr>
<tr>
<td>Azopigment fraction (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \alpha_{0F} )</td>
<td>1.08 ± 0.50</td>
<td>2.04 ± 1.00</td>
<td>1.69 ± 0.67</td>
</tr>
<tr>
<td>( \alpha_{OE} )</td>
<td>14.62 ± 1.34</td>
<td>16.29 ± 1.85</td>
<td>15.58 ± 1.22</td>
</tr>
<tr>
<td>( \alpha_{2} )</td>
<td>2.65 ± 0.39</td>
<td>2.85 ± 0.35</td>
<td>2.78 ± 0.26</td>
</tr>
<tr>
<td>( \alpha_{5} )</td>
<td>3.95 ± 0.32</td>
<td>4.07 ± 0.50</td>
<td>4.02 ± 0.36</td>
</tr>
<tr>
<td>( \beta + \gamma )</td>
<td>28.34 ± 2.50</td>
<td>23.76 ± 3.01</td>
<td>25.70 ± 2.08</td>
</tr>
<tr>
<td>( \delta )</td>
<td>50.42 ± 2.54</td>
<td>53.05 ± 2.59</td>
<td>51.94 ± 1.86</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± s.e.

Fig. 1. Inhibitory effect of 3', 4'-dideoxykanamycin B on β-glucuronidase activity.

**Effects of pH on the deconjugation of CB in bile**

Fig. 4 shows total bilirubin in bile sample against pH after 6-hr incubation. Total bilirubin concentration in both calcium bilirubinate stone group and control group was 80 to 90% of that before incubation at neutral pH (6.0-8.0). At acid pH (5.0-6.0), total bilirubin concentration in both groups apparently decreased.
Effects of pH on the Deconjugation of Conjugated Bilirubin

\[ \alpha_{OE} \] values of the control group, which were 10 to 20%, did not increase at acid to neutral pH after 6-hr incubation (Fig. 5). However, at alkaline pH they increased in a linear fashion in correlation with an increase of pH value (greater than 8.0), and reached about 30% at alkaline pH. \[ \alpha_{OF} \] values of this group (Fig. 5) also increased at alkaline pH, while they did not increase at acid to neutral pH except that one of the samples from mixed stone patients showed 10% at pH 6.0.

Fig. 2. Changes in \( \alpha_{OE} \) values of bile samples after incubation at various pH.

Fig. 3. Changes in \( \delta \) values of bile samples after incubation at various pH.
In 4 samples in the calcium bilirubinate stone group, $\alpha_{OE}$ values were the same as the controls before incubation. Increases in $\alpha_{OE}$ values which were correlated to increases of pH values were similar to those of the controls (Fig. 6). In contrast, in 2 samples in this group, $\alpha_{OE}$ values before incubation were greater than 30% and high values of $\alpha_{OE}$ were observed after incubation regardless of pH values. $\alpha_{OF}$ values in this group (Fig. 6) were less than 10% after incubation when pH value did not exceed 7.0. Between pH 7.0 and 8.0, most of them were nearly zero. However, when pH value was higher than 8.0, markedly high values of $\alpha_{OF}$ were observed in some cases, whether or not $\alpha_{OF}$ was high before incubation.

**Effects of pH on the decomposition of UCB**

UCB solutions (pH 7.6, 7.8) were incubated and total bilirubin was determined before and after incubation (Fig. 7). After 3- and 6-hr incubations at pH 7.6, total bilirubin was 83.8±1.1% and 79.5±1.2% of the preincubation value, respectively, and at pH 7.8, that was 59.1±2.9% and 39.2±1.4%, respectively.

**Effects of \(\beta\)-glucuronidase on the deconjugation of CB in bile**

Table 2 shows total bilirubin concentrations and azopigment patterns of bile samples used in the present study. When 640,000–1,280,000 Fishman units of bacterial \(\beta\)-glucuronidase was added, total bilirubin in bile samples tended to decrease after 6-hr incubation (Fig. 8). There was no statistically significant difference for the reduction of total bilirubin after incubation between the calcium bilirubinate stone group and the control group.

In all samples but one from the calcium bilirubinate stone group and one from the control group, $\alpha_{OE}$ values increased after incubation when 160,000–320,000 Fishman units of \(\beta\)-glucuronidase were added (Fig. 9). No statistically
Effects of pH on the Deconjugation of Conjugated Bilirubin

Fig. 5. pH Dependency of values of bile samples after 6-hr incubation (control group, n = 4).

Fig. 6. pH Dependency of values of bile samples after 6-hr incubation (calcium bilirubinate stone group, n = 6).
significant difference was, however, noted between these two groups.

Increases in \( \alpha_{OF} \) value were also observed in some cases when more than 320,000 Fishman units of \( \beta \)-glucuronidase were added (Fig. 9). In not only \( \alpha_{OF} \)
Effects of pH on the Deconjugation of Conjugated Bilirubin

**Table 2. Characteristics of bile samples used in the incubation with addition of β-glucuronidase**

<table>
<thead>
<tr>
<th>Total bilirubin (mg/100 ml)</th>
<th>Azopigment fraction (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\alpha_{OE}$</td>
</tr>
<tr>
<td>Calcium bilirubinate stone group</td>
<td></td>
</tr>
<tr>
<td>1*</td>
<td>17.8</td>
</tr>
<tr>
<td>2†</td>
<td>30.8</td>
</tr>
<tr>
<td>3*</td>
<td>18.8</td>
</tr>
<tr>
<td>4‡</td>
<td>12.4</td>
</tr>
<tr>
<td>5‡</td>
<td>17.0</td>
</tr>
<tr>
<td>Control group</td>
<td></td>
</tr>
<tr>
<td>6**</td>
<td>11.7</td>
</tr>
<tr>
<td>7**</td>
<td>18.9</td>
</tr>
<tr>
<td>8††</td>
<td>12.0</td>
</tr>
<tr>
<td>9††</td>
<td>13.0</td>
</tr>
</tbody>
</table>

* cholecystolithiasis; † choledocholithiasis; ‡ intrahepatic gallstones; ** malignant biliary tract disorder; †† acalculous cholecystitis; ††† cholecystocholedocholithiasis (mixed stone).

values but $\alpha_{OE}$ values, there was no statistically significant difference between two groups. However, high values of both $\alpha_{OE}$ and $\alpha_{OF}$ were only observed in some in the calcium bilirubinate stone group. In 5 of all bile samples percentages of $\delta$-fraction were less than 50% (Table 2). Most of these bile samples represented low $\alpha_{OE}$ and $\alpha_{OF}$ values after incubation with β-glucuronidase.

**DISCUSSION**

It has been suggested that there is a strong relationship between the pathogenesis of calcium bilirubinate gallstone and bacterial infection of bile (Maki et al. 1962). Formation of calcium bilirubinate gallstone is thought to be induced by stagnation of bile with ascending infection following papillitis due to mechanical and/or chemical stimulation to the papilla of Vater, migration of *Ascaris lumbricoides* into the bile duct from the papilla of Vater and so on. It is appreciated that bilirubin glucuronide in bile is hydrolyzed into UCB and glucuronic acid by β-glucuronidase, the enzymatic activity of which is increased under inflammatory conditions, and calcium combines with the liberated UCB at its free carboxyl radical, yielding the water-insoluble calcium bilirubinate. Thus enzymatic activity of β-glucuronidase in bile is presumed to be increased when calcium bilirubinate stone is formed.

Since the activity of β-glucuronidase is determined by use of an ethereal
glucuronide as substrate for assay, its increased activity would not be manifested against bilirubin glucuronide, which is an acyl glucuronide (Boonyapisit et al. 1978). Though it is necessary to utilize bilirubin glucuronide as substrate for assay, purified bilirubin glucuronide has not been available so far. Furthermore, it is also impossible to separate and purify CB in bile. Therefore, it was intended to study the decrease of CB as substrate and the increase in deconjugation products after incubation of native bile. Since native bile contains various factors concerned with the deconjugation of CB in bile, it can not be allowed to discuss about only one factor, such as enzymatic activity of β-glucuronidase, from the present results. Nevertheless, it appeared that the present experimental model faithfully reflects the reactions in vivo.

Native bile samples were incubated under conditions similar to those in vivo and changes in percentages of azopigment fractions (i.e. $\alpha_{OE}$ and $\alpha_{OF}$) during incubation were regarded as their own deconjugating indices of CB.

Ethyl anthranilate diazo reaction of bilirubin di-glucuronide provides only azodipyrrrole glucuronides (azopigment-δ) and that of bilirubin monoglucuronide, which is the main mono-conjugated bilirubin in human bile, provides azopigment-δ and unconjugated azodipyrrrole (azopigment-αΩ) (Heirwegh et al. 1974). UCB is not diazotized by ethyl anthranilate diazo reaction at pH 2.7, but

![Fig. 9] Changes of values of bile samples after 6-hr incubation with addition of β-glucuronidase (pH 7.5).

- ○ ○, calcium bilirubinate stone group (n = 5);
- • •, control group (n = 4).
Effects of pH on the Deconjugation of Conjugated Bilirubin

is diazotized p-iodoaniline and is converted into unconjugated azodipyrroles.

Increased $a_{OE}$ and $a_{OF}$ values are derived from abnormal deconjugation of CB. Based upon the data from preliminary experiments, incubation of bile samples was carried out with addition of 4 $\mu$g/ml DKB, under argon at 37°C with the protection from light. Decomposition of bilirubin was also taken into consideration.

It has been reported that pH optima of $\beta$-glucuronidase in cholesterol gallstone bile are in the range of 4.5 to 5.0 and those in calcium bilirubinate gallstone bile are in the range of 6.6 to 7.15 (Maki et al. 1962). But these experiments showed that deconjugation of CB in bile was not apparent at acid pH, which was less than 7.0, during 6-hr incubation (Fig. 5 & 6). In contrast, although it is considered that bacterial $\beta$-glucuronidase activity was low in alkaline pH, human bile showed remarkable hydrolysis in alkaline pH (8.0–9.0). And the hydrolysis was suppressed to minimum at physiologic pH (7.0–8.0), so that CB in bile was stable at physiologic pH.

After aqueous solution of UCB was incubated at pH 7.6 for 6 hours, total bilirubin concentration was kept about 80% of preincubation level. At pH 7.8 total bilirubin of UCB solution decreased to 40% of preincubation level. That means UCB is also stable at physiologic pH. These results suggested that greater extent of deconjugation of CB actually occur at alkaline pH than expected from $a_{OE}$ and $a_{OF}$ values.

Both in the calcium bilirubinate stone group and in the control group, $a_{OE}$ values did not increase and $a_{OF}$ values were less than 10% at acid to neutral pH. But at alkaline pH $a_{OE}$ and $a_{OF}$ values were high after incubation in both groups. Concerning the extent of deconjugation, there was no statistically significant difference between two groups except for one in the calcium bilirubinate stone group whose $a_{OF}$ value was higher than 20%. Cut surface of calcium bilirubinate gallstone is sometimes stratified in structure, which suggests that the formation mechanism of this type of stone may occur intermittently. Therefore, it is very important to note that extremely high value of $a_{OF}$ was seen even in only one case in the calcium bilirubinate stone group.

The experiments demonstrated that CB in bile and UCB in aqueous solution were stable at physiologic pH. Therefore, at pH 7.5, bile samples were incubated with $\beta$-glucuronidase from E. coli. In some cases UCB in bile samples after incubation increased in proportion to the enzymatic activity of $\beta$-glucuronidase, i.e. $\beta$-glucuronidase dependent hydrolysis was observed (Fig. 9). In one case no increase was seen in $a_{OE}$ and $a_{OF}$ values by addition of $\beta$-glucuronidase. This was the case with intrahepatic gallstones combined with congenital dilatation of bile duct who demonstrated low total bilirubin in bile (20.8 mg/100 ml), low percentage of $\delta$-fraction (48.0%) and high percentage of $\gamma$-fraction (24.6%) (Table 2, No. 5). In post-obstructive or cholestatic bile, the 1-O-acylglucuronide is converted into 2-, 3- and 4-O-acylglucuronides via sequential intramolecular
migrations of the bilirubin acyl group (Blanckaert et al. 1978; Compernolle et al. 1978). And \( \beta \)- and \( \gamma \)-azopigments arise from reaction of the hemiacetal hydroxy group of non C-1 glucuronides with aromatic amine (Compernolle, unpublished work). It is assumed that deconjugation of CB in bile did not occur in this case because non C-1 glucuronide is not hydrolyzed by \( \beta \)-glucuronidase. \( \alpha_{OE} \) and \( \alpha_{OF} \) values after incubation with \( \beta \)-glucuronidase were also low in cases with the percentages of \( \sigma \)-fraction less than 50\% (Table 2, No. 3, 4, 7, 9 & Fig. 9).

Compared with the control group, there was a trend of increasing hydrolysis of CB in bile in the calcium bilirubinate stone group. However, no significant difference could be demonstrated. It still remains uncertain whether or not CB in bile is more easily attacked by \( \beta \)-glucuronidase and/or others in the calcium bilirubinate stone group than in the control group. There is a possibility that bile samples (through T-tube drainage or PTBD) would have been affected by various conditions such as cholestasis, bacterial infection and outflow obstruction.

It is possible that a large amount of UCB appears in bile at alkaline pH by non-enzymatic hydrolysis of CB. At physiologic pH, however, there was no increase of UCB in bile without the enzymatic activity of \( \beta \)-glucuronidase. Therefore, it is clear that the enzymatic activity of \( \beta \)-glucuronidase is necessary for the formation of calcium bilirubinate stone following bile stasis and infection. Furthermore, other various factors may have some effects on the manifestation of its enzymatic activity. Among them effects of glucaro-1, 4-lactone, a specific inhibitor of \( \beta \)-glucuronidase (Yamaguchi et al. 1965), and bile acids are extremely important. Further investigation should be carried out for these biliary components and deconjugation of CB in bile.

**Conclusions**

Total bilirubin and azopigment pattern of native hepatic bile were determined before and after incubation at various pH and the following results were obtained:

1) CB in bile was stable at physiologic pH, but was hydrolyzed at alkaline pH even though it was not attacked by \( \beta \)-glucuronidase.

2) At pH 7.5, hydrolysis of CB in bile by \( \beta \)-glucuronidase was observed, which was demonstrated by increased \( \alpha_{OE} \) and \( \alpha_{OF} \) values after incubation.

3) As concerns hydrolysis of CB in bile, there was no statistically significant difference between the calcium bilirubinate stone group and the control group.

**References**


