EFFECT OF WHOLE BLOOD CLOTTING TIME IN RATS WITH IONIZED HYPOCALCEMIA INDUCED BY RAPID INTRAVENOUS CITRATE INFUSION

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ABSTRACT — Although the toxic effects of citrate including hemodynamic and cardiovascular changes result from a decrease in ionized calcium levels in serum due to chelating action, these effects of citrate on blood coagulation have not yet been fully clarified. The present study examines whether serum citrate and ionized calcium levels affect whole blood clotting time in rats using the test tube method in which citrate is administered by rapid intravenous infusion. Citrate was infused via the tail vein into 10 rats at 3, 4 or 5 mmol/kg/hr for 1 hr, and then whole blood clotting time, serum citrate and ionized calcium levels were determined. Whole blood clotting time did not significantly change at citrate infusion rates of 3 and 4 mmol/kg/hr. However, at 5 mmol/kg/hr, whole blood clotting time was significantly prolonged by a factor of 2.1 relative to the untreated group, when the serum citrate level was 10.03 ± 1.39 mmol/l (59.0-fold higher than that in the untreated group) and the serum-ionized calcium level was 0.29 ± 0.02 mmol/l (0.2-fold lower than that in the untreated group). These results suggest that whole blood clotting time is significantly prolonged in rats with severe ionized hypocalcemia.

KEY WORDS: Citrate infusion, Whole blood clotting time, Ionized calcium, Hypocalcemia, Rat

INTRODUCTION

Citrate has been used as an anticoagulant for blood collection and as a stabilizer in commercial intravenous solutions (for example, acetate Ringer’s solution with 1% carbohydrate and 15.5% carbohydrate solution with electrolytes). The toxic effects of citrate, including tetany, a reduction in blood pressure and prolonged electrocardiographic Q-T intervals, result from a decrease in blood ionized calcium (Ca²⁺) levels caused by its chelating action (Dzik and Kirkley, 1988; Scheidegger et al., 1980; Corbascio and Smith, 1967; Davis et al., 1995; Nakasone et al., 1954). The decreased blood Ca²⁺ level is thought to be related to the citrate infusion rate (Kahn et al., 1979), and significant changes in clinical signs, cardiovascular system and hemodynamic functions are induced at blood Ca²⁺ levels below 0.8 mmol/l in swine and humans (Liu et al., 1992; Martin et al., 1990; Olson et al., 1977).

On the other hand, Ca²⁺ is an essential cofactor for several interactions in the coagulation cascade. These include the activation of factor IX, the activation of factor X by IXa, VIa and phospholipid, the activation of factor X by tissue factor and VIIa, the cleavage of thrombin to thrombin by prothrombinase and the cross-linking of fibrin by factor XIIIa (Dzik and Kirkley, 1988). However, whether blood coagulation is affected by a decrease in the Ca²⁺ level resulting from citrate infusion has not yet been confirmed. To determine the effect of citrate on blood coagulation, the present study examined whether serum citrate and Ca²⁺ levels prolong whole blood clotting time (WBCT) in rats when citrate is rapidly infused intravenously.

MATERIALS AND METHODS

Animals and housing conditions
Six-week-old healthy male Sprague-Dawley rats

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purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan) were housed in our animal facilities until the age of 9 weeks (305-384 g). The animals were maintained in separate cages (W242 x D355 x H200 mm) at 23 ± 3°C, relative humidity of 55% ± 15%, a ventilation rate of 13-16 air exchanges/hr and a 12-hr light/12-hr dark cycle. Pelleted food (CRF-1, Oriental Yeast Co. Ltd., Tokyo, Japan) and sterilized tap water were available ad libitum. Our Institutional Committee for the Guide for the Care and Use of Laboratory Animals approved the study and all protocols proceeded in accordance with institutional guidelines.

Test solution and infusion

Anhydrous citric acid and trisodium citrate dihydrate (Wako Pure Chemical Industries, Ltd., Osaka, Japan) were dissolved in 200 ml of normal saline (Otsuka Pharmaceutical Factory, Inc., Tokushima, Japan) at a molar ratio of 1:5.25 based on CPD (citrate-phosphate-dextrose) solution (pH 5.4-5.8) (Gibson et al., 1957). Test solutions containing 50, 67 and 83 mmol/l of citrate were infused at 1 ml/kg/min for 1 hr (corresponding to citrate infusion rates of about 3, 4 and 5 mmol/kg/hr, respectively; Table 1) using a pump, into polytetrafluoroethylene catheters (24G; 0.67 mm outer diameter) placed in the tail veins of 10 conscious rats under restraint. The positive control was 0.04 ml of Novo-Heparin Injection 1000 (40 U; Novo Nordisk A/S, Denmark) dissolved in 8 ml of normal saline (Otsuka Pharmaceutical Factory, Inc.) to give a final concentration of 5 U/ml. Ten restrained, conscious rats were infused with 2 ml/min (to a dose of 50 U/kg) of heparin via a butterfly needle (24G) placed in the tail vein (Table 1). The dose of heparin required to prolong whole blood clotting time in the rats was determined from a preliminary study. Furthermore, we designated an untreated group as the control because an infusion volume of 60 ml/kg did not affect WBCT, serum Ca²⁺ levels and clinical signs, and restrained rats had already been infused with 1 ml/kg/min of saline for 1 hr in a preliminary study.

Clinical signs

Clinical signs were continuously observed during and after infusions of citrate or heparin.

Collection of blood samples

All animals were euthanized by exsanguination from the abdominal aorta following ether anesthesia and blood sample collection. Blood samples were obtained from the caudal vena cava to measure WBCT and for tests after infusion with citrate at 3, 4 and 5 mmol/kg/hr or at 3 min after infusion with heparin. Blood samples were simultaneously collected from untreated animals.

WBCT

The WBCT was measured using the test tube method that partially corrects the procedure of Lee and White (Inwood and Thompson, 1976). Briefly, equal portions of non-anticoagulated blood (2 ml) in 2 glass tubes were placed in a water bath at 37°C. From 2 min after the start of blood collection, one tube was tilted every 30 sec, whereas the other was left undisturbed. When a solid clot appeared in the first tube, the second tube was tilted every 30 sec, and the time from the start of blood collection to the time at which a clot appeared in the second tube was recorded as WBCT.

Blood test

Soon after collection for WBCT measurement, blood samples (1 ml) were placed in tubes containing a serum-separating agent, left at room temperature for 30-45 min and centrifuged at 3,000 rpm for 10 min at 4°C. Serum citrate was measured using an Automatic Analyzer 7170 (Hitachi High-Technologies Corporation, Tokyo, Japan) and serum Ca²⁺ was measured using a NOVA CRT8 (NOVA Biomedical, Waltham, MA, USA).

Table 1. Study design.

<table>
<thead>
<tr>
<th>Test solution</th>
<th>Infusion volume (ml/kg)</th>
<th>Infusion rate of citrate (mmol/kg/hr)</th>
<th>Dose of heparin (U/kg)</th>
<th>No. of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>50 mmol/l Citrate</td>
<td>60</td>
<td>3</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>67 mmol/l Citrate</td>
<td>60</td>
<td>4</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>83 mmol/l Citrate</td>
<td>60</td>
<td>5</td>
<td>-</td>
<td>10</td>
</tr>
<tr>
<td>5 U/ml Heparin</td>
<td>10</td>
<td>-</td>
<td>50</td>
<td>10</td>
</tr>
</tbody>
</table>
Statistical analysis

All data are expressed as means ± standard deviation (S.D.), and differences between the group infused with citrate and the untreated group were assessed by Dunnett’s test (Gad and Weil, 1994). Student’s t-test (Gad and Weil, 1994) assessed differences between the heparin and untreated groups and in WBCT between the heparin and the citrate (5-mmol/kg/hr) groups. A p value below 0.05 was considered statistically significant.

RESULTS

Clinical signs

Table 2 summarizes the clinical findings. None of the animals died and no changes were significant in the untreated and heparin groups. However, ptosis developed during infusion with citrate at 3 mmol/kg/hr. In addition, ptosis, tremor, hyperpnoea, face swelling and pinna reddening appeared during the infusion of citrate at 4 and 5 mmol/kg/hr. At 5 mmol/kg/hr, the rats developed tonic and clonic convulsions.

WBCT

Fig. 1 shows that the WBCT values in the untreated and citrate (3-mmol/kg/hr) groups of 5.25 ± 1.09 and 5.18 ± 0.83 min, with no significant difference. Although 4-mmol/kg/hr of citrate prolonged WBCT to 6.73 ± 0.87 min, this value did not significantly differ from that of the untreated group. In contrast, 5-mmol/kg/hr of citrate significantly prolonged WBCT compared with the untreated group, reaching 11.28 ± 2.92 min. The WBCT in the heparin group was 14.53 ± 3.94 min, which significantly differed from that of the untreated group. Although heparin tended to prolong WBCT compared with the infusion of 5-mmol/kg/hr of citrate, the difference did not reach significance.

Serum citrate and Ca²⁺ levels

Fig. 2 shows the serum citrate (A) and Ca²⁺ (B) levels, the means of which in the untreated group were 0.17 ± 0.02 and 1.37 ± 0.03 mmol/l, respectively. The serum citrate levels of animals infused with 3-, 4- and 5-mmol/kg/hr of citrate were significantly increased compared with the untreated group, reaching 2.07 ± 0.31, 5.81 ± 2.22 and 10.03 ± 1.39 mmol/l, respectively. In contrast, the serum Ca²⁺ levels were significantly decreased in these citrate groups, reaching 0.65 ± 0.06, 0.37 ± 0.05 and 0.29 ± 0.02 mmol/l, respectively. The serum citrate and Ca²⁺ levels in the heparin group were similar to those in the untreated group.

Table 2. Summary of clinical signs in male rats infused with 3, 4 or 5 mmol/kg/hr of citrate.

<table>
<thead>
<tr>
<th>Group</th>
<th>Findings</th>
<th>Before infusion</th>
<th>During and after infusions</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 mmol/kg/hr</td>
<td>Total number of animals</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Citrate</td>
<td>No. of animals with no clinical signs</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Ptosis</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>4 mmol/kg/hr</td>
<td>Total number of animals</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Citrate</td>
<td>No. of animals with no clinical signs</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Ptosis</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Tremor</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Hyperpnoea</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Face swelling</td>
<td>0</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>Pinna reddening</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>5 mmol/kg/hr</td>
<td>Total number of animals</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Citrate</td>
<td>No. of animals with no clinical signs</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Ptosis</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Tremor</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Hyperpnoea</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Face swelling</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Pinna reddening</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Tonic convulsion</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Clonic convulsion</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Heparin</td>
<td>Total number of animals</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>No. of animals with no clinical signs</td>
<td>10</td>
<td>0</td>
</tr>
</tbody>
</table>

Note. No changes in clinical signs were evident in the untreated group.
DISCUSSION

Standard tests of blood coagulation such as the prothrombin and activated partial thromboplastin times appear not to reflect the effects of decreased Ca\(^{2+}\) resulting from citrate infusion because reagent calcium is added to test samples at the start of the test. Therefore, we used the test tube method to measure WBCT in rats that had received rapid infusions of citrate.

Citrate infusions at 3 and 4 mmol/kg/hr significantly decreased serum Ca\(^{2+}\) levels to 0.65 ± 0.06 (0.5-fold less than that in the untreated group) and to 0.37± 0.05 mmol/l (0.3-fold less than that in the untreated group), respectively. However, WBCT did not significantly change at 3 and 4 mmol/kg/hr of citrate despite the appearance of clinical signs that are considered to be related to ionized hypocalcemia (Kishimoto et al., 2002; Fukuda et al., 2002). In addition, electrocardiographic changes such as prolonged Q-T intervals and T wave alternans or depressions in arterial pressure and systemic vascular resistance are induced at above similar blood Ca\(^{2+}\) levels in studies with pigs, dogs or humans (Dzik and Kirkley, 1988; Scheidegger et al., 1980; Liu et al., 1992; Olson et al., 1977; Howland et al., 1977; Bunker et al., 1962; Drop and Scheidegger, 1979). Thus, the effect on WBCT caused by a decrease in serum Ca\(^{2+}\) is considered minimal compared with cardiovascular and hemodynamic effects as well as clinical signs. On the other hand, citrate infused at 5 mmol/kg/hr significantly prolonged WBCT (2.1-fold longer than that in the untreated group) to a level comparable with that of the positive heparin control, caused tonic and clonic convulsions, significantly increased serum citrate to 10.03 ± 1.39 mmol/l (59.0-fold higher than that in the untreated group) and significantly decreased serum Ca\(^{2+}\) to 0.29 ± 0.02 mmol/l (0.2-fold lower than that in the untreated group). Incidentally, platelet adherence to the subendothelial matrix via von Willebrand’s protein requires a concentration of at least 0.3 mmol/l Ca\(^{2+}\) (Sakariassen et al., 1984), which is close to the serum level of Ca\(^{2+}\) induced by 5-mmol/ kg/hr of citrate. Furthermore, WBCT was obviously prolonged in this group. Future studies should determine whether abnormalities in platelet function and bleeding time are induced by ionized hypocalcemia resulting from citrate infusion.

In clinical practice, a significantly prolonged WBCT or other types of hypocalcemic toxicity induced by citrate might be preventable if pertinent calcium therapy is applied during infusions of citrated blood products (Martin et al., 1990; Liu et al., 1992; Weinstein, 1996). In addition, Ca\(^{2+}\) is a component of

![Fig. 1. WBCT in male rats infused with 3, 4 or 5 mmol/kg/hr of citrate. Values are means ± S.D. of 10 rats. Significantly different from untreated group: *** p < 0.001 (Dunnett’s test) and ***, p < 0.001 (Student’s t-test). No difference was significant between groups infused with 5-mmol/kg/hr of citrate or heparin (Student’s t-test).](image-url)
intravenous products such as acetate Ringer’s solution with 1% carbohydrate (Trade name: Physio® 140, Otsuka Pharmaceutical Factory, Inc.) and 10.5% carbohydrate with electrolytes (Trade name: TRIFLUID®, Otsuka Pharmaceutical Factory, Inc.), suggesting that citrate might not induce toxic effects in clinical practice.

In conclusion, WBCT was significantly prolonged in rats infused with citrate at 5 mmol/kg/hr for 1 hr. The serum citrate and Ca²⁺ levels required to pro-

![Graph A](image1)

(A)

![Graph B](image2)

(B)

Fig. 2. Serum citrate (A) and Ca²⁺ (B) levels in male rats infused with 3, 4 or 5 mmol/kg/hr of citrate.

Values are means ± S.D. of 10 rats. Significantly different from untreated group: **, p < 0.01 and ***, p < 0.001 (Dunnett’s test). No differences were significant between the untreated group and the heparin group (Student’s t-test).
long WBCT in rats are therefore 10.03 ± 1.39 and 0.29 ± 0.02 mmol/l, respectively.

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REFERENCES


