THROMBOSIS RESEARCH 24; 505-508, 1981
0049-3848/81/230505-04$02.00/0

METHOD: MINI-REPORT

3.8% SODIUM CITRATE (1:9) IS AN INADEQUATE ANTICOAGULANT FOR RABBIT BLOOD WITH HIGH CALCIUM

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(Received 1.10.1981; in revised form 10.11.1981.
Accepted by B. Blombäck)

INTRODUCTION

Sodium citrate, 3.8 g/dl (129 mmol/l), is a widely used anticoagulant for blood collection for clinical laboratory analyses. The use of the same concentration to anticoagulate blood drawn from New Zealand White rabbits was not uniformly successful in preventing fibrin formation. The following report documents higher concentrations of total and ionized calcium, lower albumin and total protein concentrations, and lower hematocrit in rabbit blood. Nascent fibrin formation in response to thromboplastin challenge is demonstrated when traditional amounts of citrate are used.

METHODS

A two syringe technique was used for all blood drawing. Serum calcium concentration was determined by atomic absorption spectroscopy. Serum protein and albumin were assayed by the biuret reaction without and with sodium sulfate precipitation, respectively. Ionized calcium was determined by an ion specific electrode using an Orion Model SS-20 system for whole blood anticoagulated with 5 units/ml porcine heparin. For the titration of calcium chloride with sodium citrate, 9 parts heparinized whole blood was mixed with 1 part sodium citrate to yield the plotted final citrate concentration of the specimen. Aerobic conditions were used for the titration.

A protamine sulfate paracoagulation test was done by the method of Niewiarowski and Gurewich (1) to assess anticoagulation. Platelet poor plasma was prepared from 9 parts blood added to 1 part sodium citrate (9:1) to yield the final citrate concentration of 12.9 mmol/l for the human specimens and 12.9, 15, 17.5, and 20 mmol/l for rabbit samples. A controlled amount of Dade activated rabbit brain thromboplastin solution, 20 µl, was added to 1 ml platelet poor plasma in a glass tube and stored for 30 min at 22 °C, 20 hours at 4 °C, or 20 hours frozen with a 1 hour thawing period at 22 °C. Two hundred microliters plasma and 200 µl Tris buffered 0.1 g/dl protamine, pH 6.5, were

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Key words: anticoagulant, ionized calcium, rabbits, serum calcium, sodium citrate

incubated at 22 °C for 30 min in glass tubes. A score of 1 was given for a feathery precipitate, a score of 2 for fibrin strands, and a score of 3 for gel formation.

Statistical comparisons were made with the Student t test for unpaired data. Mean ± standard error of the mean are used throughout.

RESULTS

Titration of an aqueous calcium chloride solution with sodium citrate (fig. 1, curve 1) demonstrates the one mole calcium to 2/3 mole citrate stoichiometry and a dissociation constant for the complexed calcium between 10^{-3} and 10^{-2}. Accuracy of the electrode determination at low ionized calcium concentrations was experimentally verified. In human whole blood (fig. 1, curve 2) the initial stoichiometric ratio is approximately 1 mole calcium to 3.2 moles citrate. This is similar to the 3 to 10 ratio noted 60 years ago by Vines (2) using a clotting endpoint. For rabbit blood (fig. 1, curve 3) the initial ratio is 1 mole calcium to 2.5 moles citrate. Presumably, since the citrate anion added to whole blood complexes with other cations and cationic sites of large molecules, the decrease in ionized calcium for blood is achieved with much more citrate than for the aqueous calcium chloride titration.

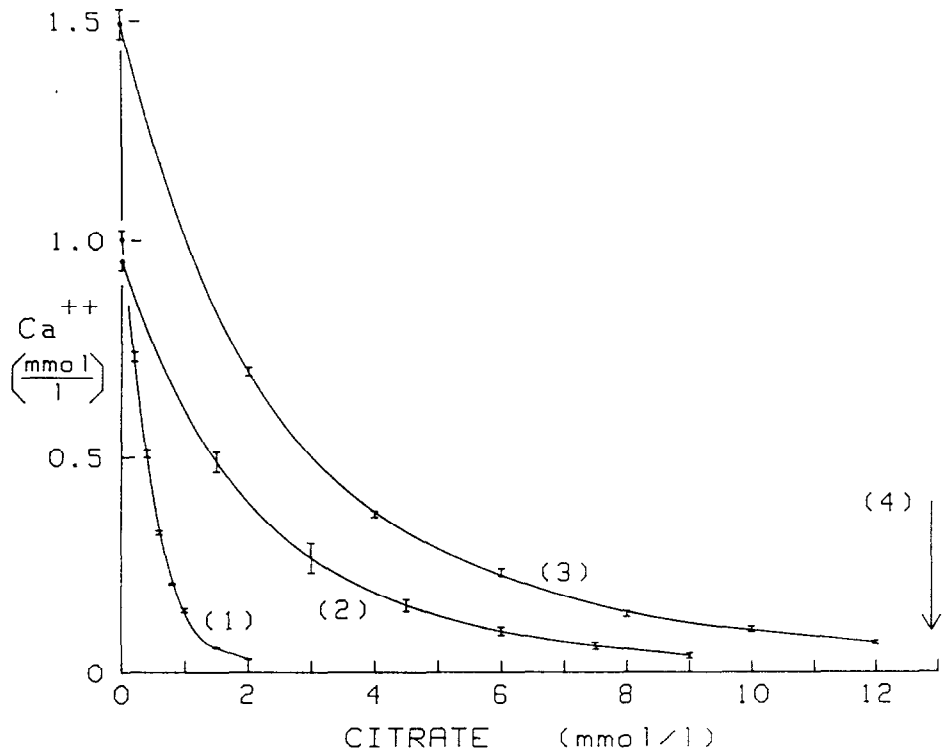

FIG. 1
Sodium citrate titration of ionized calcium for (1) an aqueous solution of calcium chloride, (2) human whole blood, and (3) rabbit whole blood. The traditional concentration of citrate (1 part 3.8 g/dl sodium citrate to 9 parts whole blood) is denoted by (4).

The minimum ionized calcium sufficient for clotting reported by Ransmeier and McLean (3) is 0.28 mmol/l. This concentration is reached with only 2.9 mmol/l citrate for human blood and 5.3 mmol/l citrate in rabbit blood. The achievement of low ionized calcium with an amount of citrate insufficient to prevent clotting is consistent with earlier conclusions that the state of bound calcium is also important in anticoagulation (2,5).

The 44% greater total serum calcium concentration for rabbits (table 1) is only partially explained by the ionized pool which is 32% greater. The nonionized calcium must also increase inspite of 18% less protein. Assuming that approximately five percent of calcium is complexed with nonprotein organic molecules in rabbit blood as in human blood, then the protein binding must nearly double, 15 micromoles calcium per gram protein in humans compared with 28 μmol/g in rabbits. If sodium citrate were increased proportionally for the increased ionized calcium and lower hematocrit in rabbit blood, one part 191 mmol/l sodium citrate to 9 parts blood would be predicted to be adequate. Based on the increased total calcium 208 mmol/l sodium citrate would be predicted for an effect comparable to 129 mmol/l used for human blood.

TABLE 1
Comparison of Human and Rabbit Calcium and Protein Concentrations

	Human (n=10)	Rabbits (n=10)	p value
Calcium, total (mmol/l)	2.31 ± .03	3.32 ± .08	< .0001
Calcium, ionized (mmol/l)	1.04 ± .01	1.37 ± .02	< .0001
Ionized calcium (% of total)	45.2 ± 0.4	41.6 ± 0.8	.0009
Total protein (g/dl)	7.74 ± .14	6.32 ± .09	< .0001
Albumin (g/dl)	4.84 ± .10	3.82 ± .04	< .0001
Albumin/globulin ratio	1.69 ± .08	1.54 ± .06	.17
Hematocrit	45.7 ± 1.2	39.1 ± 1.2	.0012

Since the first indication of the possible inadequacy of 129 mmol/l (3.8 g/dl) sodium citrate (1:9) for rabbit blood anticoagulation was related to difficult venapuncture and tissue thromboplastin production, we subsequently challenged the adequacy of anticoagulation of rabbit blood with thromboplastin. The protamine sulfate paracoagulation test has been shown by Kidder (4) to be capable of detecting nascent clotting. Table 2 shows the sharp difference between the traditional (129 mmol/l, 1:9 mixture) citrate anticoagulation used for rabbit blood compared with human blood. At 20 mmol/l final citrate concentration the protamine sulfate paracoagulation test did not demonstrate clotting. This concentration is close to that predicted by a proportional increase in citrate based on the measured calcium increase and hematocrit decrease in the rabbit blood.

TABLE 2
Paracoagulation after Addition of Thromboplastin to Plasma
Anticoagulated with Varying Concentrations of Sodium Citrate (n=3)

Final citrate conc. (mmol/l)	Human 12.9	Rabbit 12.9	Rabbit 15.0	Rabbit 17.5	Rabbit 20.0
2 hours at 22° C.	0	1.0	0.7	0	0
20 hours at 4° C.	0	1.3	1.3	0.7	0
20 h frozen + 1 h thaw	0	1.3	1.0	1.0	0

Of the reports published in Thrombosis Research in 1979 and 1980 which used rabbit blood in vitro and stipulated the anticoagulant used, 25 reports used a calcium chelating anticoagulant. Five reports used less than 12.9 mmol/l citrate final concentration, 11 used 12.9 mmol/l citrate, and 5 used more than 12.9 mmol/l citrate. Four reports used ethylenediaminetetraacetic acid chelation of calcium.

DISCUSSION

With careful blood collection to minimize tissue thromboplastin, anticoagulation of rabbit blood 9 parts to 1 part 129 mmol/l (3.8 g/dl) sodium citrate may be sufficient for short time durations. However, we have found the use of 200 mmol/l sodium citrate solution to be more reliable. Use of acid-citrate-dextrose solution, 113 mmol/l citrate, or citrate-phosphate-dextrose solution, 105 mmol/l citrate, are even more likely to be inadequate unless more than 1 part anticoagulant solution to 9 parts whole blood are used. Over 30 years ago, Quick (5) noted the higher concentration of sodium citrate necessary in studies on rabbit prothrombin and suggested that 20 mmol/l (final concentration) was necessary. Even when fibrin formation is not evident, as is usually the case, the higher concentrations of calcium may result in erroneous conclusions when techniques validated with human material are applied to rabbit plasma or platelet suspensions. At the present time additional citrate is not commonly used for the anticoagulation of rabbit blood.

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