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Title: Increase in serum potassium levels after refrigeration storage: a component of blood clot contaminates the serum layer over the separator gel

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Key words: Refrigeration, blood collection, serum potassium, blood clots, haemolysis

Abstract

Objectives: To determine the cause of elevated serum potassium levels when blood collection tubes containing separator gel are stored under refrigeration.

Methods: Fifty-seven hospitalized patients and 11 healthy volunteers were recruited. Venous blood samples were obtained using Insepac II, Neotube, and Venoject® II, without anticoagulant. After centrifugation under different processing conditions, the capped tubes were stored at 4°C without aliquoting, and serum potassium levels were measured for up to 14 days. The correlation between the increase in potassium levels and blood cell counts was assessed. Furthermore, the serum was replaced with a saline solution and potassium levels were determined after refrigeration.

Results: Refrigerated samples stored in Insepac II tubes had significantly higher serum potassium levels on day 14 than on the day of blood collection. The increase in serum potassium levels was positively correlated with the number of red blood cells but not white blood cells and platelets in venous blood. Furthermore, the potassium levels were elevated when the serum was replaced with a saline solution. Using Venoject® II, which has a larger tube diameter and a thicker separator gel than those of Insepac II and Neotube, did not increase the serum potassium levels after storage. The increase in the serum potassium level was markedly suppressed by centrifugation at 2330 × *g* for 15 min relative to other processing conditions.

Conclusions: Potassium levels increase when the serum is refrigerated in collection tubes containing separator gel. This can be attributed to contamination of the serum layer by blood cell components beyond the separator gel.

Introduction

Serum specimens are usually stored in clinical laboratories where additional tests may be requested later. This is convenient because it eliminates the need to re-collect blood from the patient. Conversely, the gold standard for achieving this objective is to aliquot and cryopreserve serum for different tests. However, the rules for specimen preservation are not uniform and vary among facilities.

Serum gel tubes are shown to be more beneficial than plain serum and plasma tubes for specimen storage [1], and most serum components showed no change for about 14 days after refrigerated storage [2, 3]. Particularly, Ikeda et al. [3] dispensed the serum into multiple tubes, stored them at a given temperature, and evaluated the changes in each constituent. While this method can correctly assess changes in serum samples over time, it is difficult to implement such a method for multiple samples. In practice, facilities refrigerate serum in serum gel tubes without dispensing it into other tubes. Here, serum and blood cells coexist in the same tube, separated by a separating agent.

Potassium ion (K^+) exists in greater amounts intracellularly than extracellularly, and haemolysis leads to a falsely positive impact [4]. Therefore, serum potassium in blood samples is unstable and samples should be centrifuged soon after blood collection. After serum separation, samples can be stored for 6 weeks if kept refrigerated [5]. Nevertheless, Dimenski et al. [6] showed an increase in serum potassium levels under prolonged storage of the tubes for 168 h at 2–8℃; however, the cause is unclear. In this study, we focused on the increased serum potassium when blood collection tubes containing the serum gel separator were refrigerated. We used methods simulating real-world conditions that could be implemented for specimen storage in hospitals. We hypothesise that this phenomenon is caused by the haemolysis of red blood cells (RBCs) and aim

to clarify the cause as (i) the RBCs in the serum or (ii) the blood cell layer (blood clots) below the separating agent. We discovered that this could be attributed to contamination of the serum layer by blood cell components beyond the separator agent.

Materials and Methods

Subjects

Fifty-seven hospitalized patients [68 ± 15 years old (years ± standard deviation)] receiving medical care at Hyogo Prefectural Rehabilitation Center at Nishi-Harima in Japan, and 11 healthy volunteers [40 ± 7 years old] were included (Table 1 and Supplementary Table 1). All study participants provided informed consent. The study was approved by the ethics committee of the Hyogo Prefectural Rehabilitation Hospital at Nishi-Harima (No. 2022), and performed in accordance with the principles of the Declaration of Helsinki.

Serum isolation and measurement of potassium and sodium levels

Venous blood samples were obtained using Insepac II (SMD750SQ; Sekisui Medical, Japan), Neotube (NP-SP0725; Nipro, Japan), and Venoject® II (VP-AS104K; TERUMO, Japan), without anticoagulant, as appropriate. These were mixed by inverting, left to stand at room temperature (between 15°C and 25°C) for at least 30 min, and then centrifuged at 2330 × *g* for 7 min. Primary serum potassium and sodium levels were measured using an automatic biochemical analyser (TBA-120FR; Canon Medical Systems, Japan) (T0d, used for reference measurement). Thereafter, the capped tubes were stored at 4°C without aliquoting, and the same parameters were measured after 1, 3, 7, and 14 days (T1d, T3d, T7d, and T14d, respectively). The samples were neither haemolysed nor icteric or lipemic.

RBCs count in serum

Venous blood samples were collected using Insepac II tubes. The serum was separated by centrifugation using the method described above (2330 × *g* for 7 min), poured directly into a new conical tube. To collect all the RBCs remaining above the separator, 2 mL of fresh saline was added to the Insepac II tube, mixed by gently inverting, and then poured into the conical tube. The above steps were repeated, and the total volume (almost 6 mL) of the sample in the conical tube was centrifuged at 1400 × *g* for 5 min. The supernatant was discarded, the pellet was suspended in 1 mL of saline, and the number of cells was counted using a Fuchs-Rosenthal counting chamber (Supplementary Figure 1).

Measurement of potassium concentration in RBCs

Blood was drawn from a vein using a heparinized blood collection tube (BD Vacutainer, Becton, Dickinson and Company), and the RBC count was measured using an automated haemocytometer (XN-1000; Sysmex, Japan). Further, the erythrocytes were diluted with saline and subjected to freeze-thaw at –30°C to haemolyse the erythrocytes. The potassium level in RBCs was measured using TBA-120FR.

Evaluation of the increase in potassium under different centrifugal conditions

Venous blood was collected in a blood collection tube containing a separator, and centrifuged under the specified conditions (1500 × *g* for 10 min or 15min; 1710 × *g* for 10 min or 15 min; or 2330 × *g* for 7 min, 10 min, or 15 min) using a centrifuge (5420; KUBOTA, Japan). Serum potassium levels were measured using a TBA-120FR clinical chemistry analyzer at T0d and T14d. Capped sample tubes were stored upright at 4°C.

Replacement of serum with a saline solution

Blood was collected intravenously into a blood collection tube containing a separator and centrifuged under the specified conditions (1710 × *g* for 10 min, and 2330 × *g* for 7 min, 10 min, and 15 min). Because the RBCs remaining in the serum at the top of the separator must be completely haemolysed, the serum layer was discarded, replaced with 2mL sterile water, mixed, and allowed to stand for 6 h at room temperature. The upper layer was discarded, and then 2mL saline solution was added to the tubes. The upper layer was used as the sample. The samples were then stored at 4°C for 14 days, and potassium levels were measured using TBA-120FR (Supplementary Figure 2).

Cell count in venous blood

Blood was collected in an ethylenediaminetetraacetic acid EDTA-2K blood collection tube (BD vacutainer, Becton, Dickinson and Company), and RBCs, white blood cells (WBCs), and platelets (PLTs) counts were estimated using XN-1000.

Statistical analysis

The correlation coefficient '*r*' was evaluated using the Pearson correlation method. We also assessed statistical significance using the Student t test, Dunnett's test or Tukey's test, as appropriate and considered a probability of < 5% ($P < 0.05$) to be statistically significant. The statistical analyses were performed using EZR software version 1.36, based on R and R commander [7].

Results

Serum potassium levels change after refrigerated storage

We first examined the changes in serum components after refrigerated storage (up to T14d) in blood collection tubes without dispensing serum. Here, the serum potassium levels were significantly increased compared to that at T0d. To assess whether this increase was due to enrichment, changes in sodium levels of the same sample were examined. There was no change in serum sodium levels (Fig. 1) or the other parameters (data not shown). We next expected that the serum layer was not sufficiently separated due to inadequate coagulation caused by the antithrombotic medication, leading to an increase in serum potassium after refrigeration storage. However, the increase in serum potassium levels after refrigerated storage was stronger in non-users than in users of antithrombotic drugs, both groups showed a clear increase in serum potassium levels after refrigerated storage (Supplementary Figure 3). Therefore, this phenomenon may be independent of enrichment by evaporation and antithrombotic medications, however, these results need further validation due to the small sample size.

Relationship between the increase in serum potassium levels and the number of serum RBCs

Next, we hypothesized that the increase in serum potassium levels during refrigerated storage was due to the haemolysis of RBCs. We confirmed the presence of fresh RBCs after centrifuging the serum again (Fig. 2A); however, there was no correlation between the amount of increase in serum potassium levels and the number of serum RBCs ($r = 0.014$, Fig. 2B). Furthermore, heparinized blood was serially diluted to determine the amount of potassium due to erythrocyte haemolysis. The potassium value (mmol/L) was expressed as [0.0075 × erythrocyte count (cells/mL) − 0.014] (Fig. 2D). Because the mean serum RBC count was almost 20×10^6 cells (Fig. 2C), even when

all of the cells were haemolysed, the calculated potassium levels due to haemolysis were less than 0.15 mmol/L (Fig. 2D). In this experiment, RBCs were suspended in 1 mL of saline, but assuming that the Insepac II blood collection tube could collect approximately 2 mL of serum, the levels of potassium would have been further reduced. These results suggest that the increased serum potassium levels were not due to the haemolysis of RBCs in the serum.

Relationship between the increase in serum potassium levels and the number of RBCs in venous blood

We further speculated that a part of the blood cell layer (blood clots) might have passed through the separator gel into the serum, leading to an increase in serum potassium. Indeed, there was a positive association between the amount of increase in serum potassium levels and the number of RBCs ($r = 0.366$), but not WBCs and PLTs in venous blood ($r = 0.014$ and $r = 0.044$, respectively) (Fig. 3).

Conditions that prevent an increase in serum potassium levels after refrigerated storage

Next, we examined whether the increase in serum potassium level could be suppressed by changing the centrifugation conditions from 2330 × *g* for 7 min to 1500 × *g*, 1710 × *g,* or 2330 × *g* for 10 min or 15 min. The increase in the serum potassium level was markedly suppressed by centrifugation at 2330 × *g* for 15 min compared to other conditions including 2330 × *g* for 7 min and 10 min, and 1500 × *g* for 10 min (Fig. 4A). Next, the serum on top of the separator was discarded, the sample was washed, and the serum was replaced with a saline solution, to examine whether the components of the blood cell layer exceed the separating agent. The sample was then refrigerated for 14 days, and the change in potassium level was measured. Although nothing abnormal was observed immediately after washing, after 14 days of refrigerated storage,

haemoglobin pigment was visually observed under the separator gel (Fig. 4B). The potassium concentration in this sample was increased, but the increase was suppressed in the sample centrifuged at 2330 × *g* for 15 min (Fig. 4C). Therefore, we focused on the amount of separator gel in blood collection tubes (Fig. 4D). Similar to Insepac, mainly used in this study, the increase in serum potassium levels after refrigerated storage was also observed in Neotube, in which the thickness of the separator gel was comparable to that of Insepac. In contrast, samples stored in Venoject® II tubes, in which the diameter of the tube was wider and the separator gel layer was thicker than those of Insepac and Neotube tubes, did not show an increase in the serum potassium levels (Fig. 4E).

Discussion

Serum potassium levels increase when serum is refrigerated in the blood collection tubes. In this study, we showed that this increase is caused by the mixing of blood components from the blood cell layer with the serum beyond the separating agent.

On the other hand, Ikeda et al. [3] reported that serum potassium levels did not change after refrigerated storage for up to 14 days. Serum components were evaluated over time by dispensing and freezing serum into separate containers, dissolving it for each measurement, and quantifying it at each time point [3]. This method can evaluate the changes in serum components only; however, in actual clinical laboratories, the serum is often stored in vacuum collection tubes without dispensing.

Conversely, Kift et al. [8] reported a significant increase in serum potassium after 4 days of storage at 4°C without a lid. An increase in sodium levels was also observed; hence we consider this to be a significant effect of enrichment due to evaporative concentration. In contrast, we found that the serum potassium level increased over time while that of sodium remained unchanged

when the tubes were kept refrigerated with the lid on and not aliquoted. The difference between our storage conditions and that of the others is that the serum is not dispensed into a separate container but is stored in the blood collection tube without dispensing.

Therefore, we first speculated that the cause of the increased serum potassium level is the haemolysis of RBCs. We expected haemolysis of RBCs in the serum. However, when the number of RBCs and the corresponding change in the potassium level were assessed, the actual increase in potassium level was not found. Moreover, there was no correlation between the number of RBCs in the serum and the increase in potassium. Therefore, we inferred that the increase in serum potassium was not due to haemolysis of the RBCs in the serum.

Next, we focused on the blood cell layer that existed below the separator. A positive correlation was found between whole blood RBC count and increased serum potassium levels, but not WBCs and PLTs. Furthermore, we then centrifuged venous blood, replaced the serum layer above the separator with a saline solution, and measured the potassium levels after refrigerated storage for 14 days. We could not visually observe any obvious change in the top of the separating gel after refrigeration for 14 days after the replacement of the saline solution, however, potassium was detected. This suggested that the increased serum potassium level was due to contamination from the blood cell layer beyond the separating agent into the serum.

It remains unclear why this phenomenon occurs in this study. We speculate, however, that some of the components that should have been separated as serum remain in the blood cell layer due to the rather low centrifugal strength (e.g., 2330 × *g* for 7 min). Consequently, these components exceed the separating gel and enter the serum layer, while potassium released by haemolysis in the blood cell layer during refrigeration may have entered the serum layer simultaneously. Practically, the length of the serum layer was significantly greater when centrifuged at 2330 × *g* for 15 min than at 2330 × *g* for 7 min, and conversely, it was significantly less in the clot layer

(Supplementary Fig. 4). This result suggested that the centrifugal condition of 2330× *g* for 7min is insufficient for the amount of serum that should be obtained.

Measures taken to control this phenomenon showed that the increase in potassium levels could be suppressed by performing centrifugation and by selecting blood collection tubes that contained a large amount of separating agent. The materials of serum separators include acrylic, polyolefin, and polyester, and these compounds affect blood drug concentrations [9,10]. The three types of blood collection tubes used in this study were polyolefin-based for Insepac and Venoject, and polyester-based for Neotube. In this study, the increase in serum potassium levels on refrigeration was observed for Insepac and Neotube, but not for Venoject, suggesting that this phenomenon was not due to the material of the serum separator. Furthermore, in this study, the centrifugal condition of 2330 × *g* for 15 min was effective in suppressing the increase of potassium level after refrigeration storage. Although WHO guidelines recommend centrifuging the sample for at least 10 min at a minimum speed of 1500 × *g* [5], in this study the potassium level was significantly increased after 14 days of refrigeration storage as compared with 2330 × *g* for 15 min (Fig. 4A). Further studies are needed to determine if this phenomenon occurs with other acrylic blood collection tubes and centrifugation conditions.

Similar to potassium, LDH, especially LDH1 and LDH2, are clinical chemistry parameters that are increased by haemolysis [11]. Although isozyme analysis was not performed in this study, it is possible that these also increase with refrigeration, and we plan to assess the effect of refrigeration on isozyme levels in future studies.

This study has some limitations. The sample size for this study was small. We believe that further study needs to be conducted with a larger sample size. The centrifuge we used in this study does not have a cooling function, and we did not examine the increase in chamber temperature due to increased centrifugation time. However, since the increase of serum potassium values after

refrigeration storage was suppressed by the strongest centrifugal conditions, we think that the temperature in the centrifuge had little effect on this phenomenon. We have not included all blood collection tubes in our study. The material and volume of the serum separator in blood collection tubes varies among manufacturers, and the tubes used vary among the facilities. In addition, there were seven different centrifugal conditions and times tested in this study, respectively. In this study, we concluded that 2330 × *g* for 15 min was the most ideal among them, but more suitable conditions may be found by setting detailed conditions. It is necessary to determine the extent to which the results of this study, i.e., the increase in serum potassium levels due to refrigerated storage, occur depending on the tubes and centrifugal conditions used at each facility.

In conclusion, we showed that serum potassium levels increase when serum is refrigerated in the collection tube, which can be attributed to the contamination of blood cell components in the serum beyond the separator gel.

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Author contribution

All authors were involved in drafting the article, revising it critically for important intellectual

content. All authors approved the final version of the manuscript to be published. KY has full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study concept and design: KY. Acquisition of data: KY, HT, SN, SY. Analysis and interpretation of data: KY.

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Table 1. Profile of the participants in this study.

	Patients (N = 57)	Healthy volunteers (N = 11)
Mean age (± SD)	68 (15)	40 (7)
Male ratio (%)	32 (56.1)	9 (81.8)
Hypertension (%)	24 (42.1)	0 (0)
Dyslipidaemia (%)	5 (8.8)	0 (0)
Diabetes mellitus (%)	10 (17.5)	0 (0)
Use of antithrombotic agents (%)	15 (26.3)	0 (0)

SD: standard deviation.

Figure legend

Figure 1. Change in serum potassium/sodium levels (mmol/L) (left) and rate of change (%) (right) from the day of blood collection (T0d) to T14d after refrigeration storage (N = 5). Values shown are means ± standard deviation (SD). Statistical significance was determined by using Dunnett's test. **P* < 0.05 (versus serum potassium levels at T0d). ***P* < 0.05 (versus the rate of change from T0d to T14d in serum sodium levels).

Figure 2. Relation between the increased serum potassium levels from the day of blood collection (T0d) to T14d and the number of red blood cells (RBCs) in serum.

(A) Erythrocytes present in serum (×400)

(B) Correlation between the amount of change in serum potassium levels from T0d to T14d and the number of RBC in serum on T0d (N = 7).

(C) Correlation between the number of erythrocytes in venous blood and serum on T0d (N = 36).

(D) Relationship between the number of RBCs and the potassium level after complete haemolysis.

Figure 3. Correlation between the amount of change in serum potassium levels from T0d to T14d and the number of venous blood cells [(RBCs, white blood cells (WBCs), and platelets (PLTs)] at T0d (N = 57). Statistical significance was determined using the Pearson correlation method. **P* < 0.05.

Figure 4. Differences in the increase in serum potassium levels due to centrifugation and type of blood collection tube. The experiments were conducted using healthy volunteers, values shown are means ± standard deviation (SD), and statistical significance was evaluated using Tukey's test. **P* < 0.05.

(A) Rate of Change over time in serum potassium levels from the day of blood collection (T0d) to T14d after different centrifugation conditions and refrigerated storage (N = 8).

(B) Representative image in blood collection after the replacement of serum with a saline solution after centrifugation at 2330 × *g* for 7 min, 10min, 15min, and 1500 × *g* for 10 min (from T0d to T14d). Arrows indicate haemoglobin pigment is visible.

(C) Increase in potassium levels in blood collection tubes at T14d after replacement of serum with a saline solution and refrigerated storage (N = 5).

(D) Representative image in blood collection tubes, Insepac II, Neotube, and Venoject II after centrifugation at 2330 × *g* for 7 min (T0d).

(E) Rate of Change in serum potassium levels from T0d to T14d after centrifugation under 2330 × *g* for 7 min in different types of blood collection tubes and refrigerated storage (N = 7).

Supplementary Figure 1.

Venous blood samples were obtained using Insepac II tubes. The serum was separated by centrifugation using the method described above (2330 × *g* for 7 min), poured directly into a new conical tube. To collect all RBCs remaining above the separator, 2mL of fresh saline was added to their Insepac II tube, mixed by gently inverting, and then poured into the conical tube. The above steps were repeated, and the sample's total volume (almost 6mL) in conical tubes was centrifuged at 1400 × *g* for 5 min. The supernatant was discarded, the pellet was suspended in 1 mL of saline, and the number of cells was counted using a Fuchs-Rosenthal counting chamber.

Supplementary Figure 2.

Blood was collected intravenously into a blood collection tube containing a separator and centrifuged under the specified conditions (1710 × *g* for 10 min, and 2330 × *g* for 7 min, 10 min,

and 15 min). Because the RBCs in the serum that remain at the top of the separator must be completely haemolyse, the serum layer was discarded, replaced with sterile water, mixed, and allowed to stand for 6 h at room temperature. The upper layer was discarded, and then the saline solution was added to the tubes. The upper layer was used as the sample. The samples were then stored at 4°C for 14 days, and potassium levels were measured using TBA-120FR.

Supplementary Figure 3.

Rate of change (%) in serum potassium levels from the day of blood collection (T0d) to T14d after refrigeration storage in patients on antithombotic drugs (N = 15) or not (N = 42).

Supplementary Figure 4.

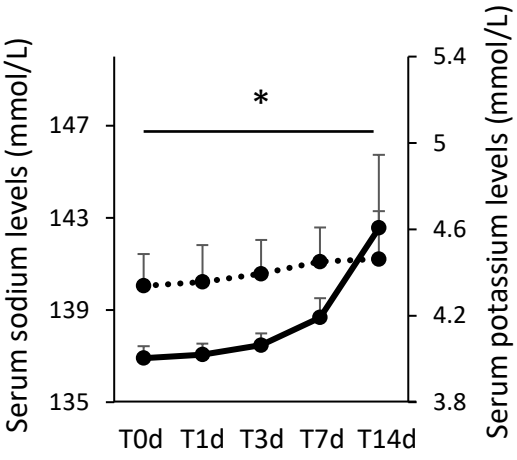
Differences in the separation of serum and blood cell layers under different centrifugal conditions (N = 5).

(A) Schematic image in this experiment. We centrifuged venous blood at 2330 × *g* for 7 min, and 15 min: the maximum blood cell layer diameter was (a, mm), and the minimum blood cell layer diameter was (b, mm), and the serum layer diameter was (c, mm),

(B) Representative data in blood collection tube after centrifugation at 2330 × *g* for 7 min, and 15 min.

(C) The length of the serum layer was significantly greater when centrifuged at 2330 × *g* for 15 min than at 2330 × *g* for 7 min, and conversely, it was significantly less in the clot layer. Values shown are means ± standard deviation (SD). Statistical significance was determined by using the Student t test. **P* < 0.05.

Fig1


Serum sodium levels (mmol/L)
147
143
139
135
*
5.4
5
4.6
4.2
3.8
Serum potassium levels (mmol/L)
T0d T1d T3d T7d T14d

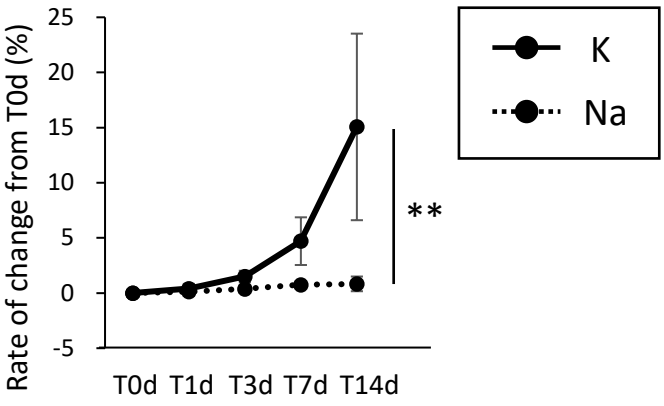
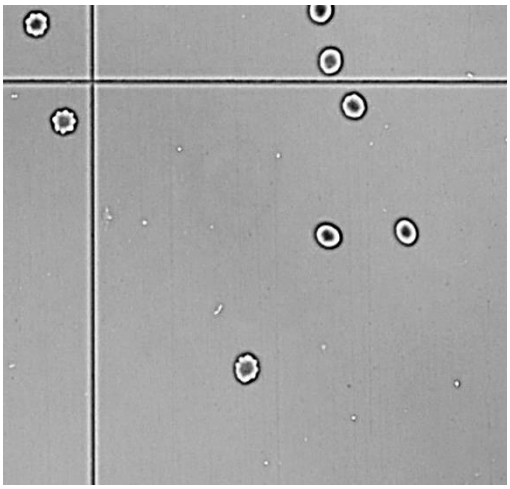
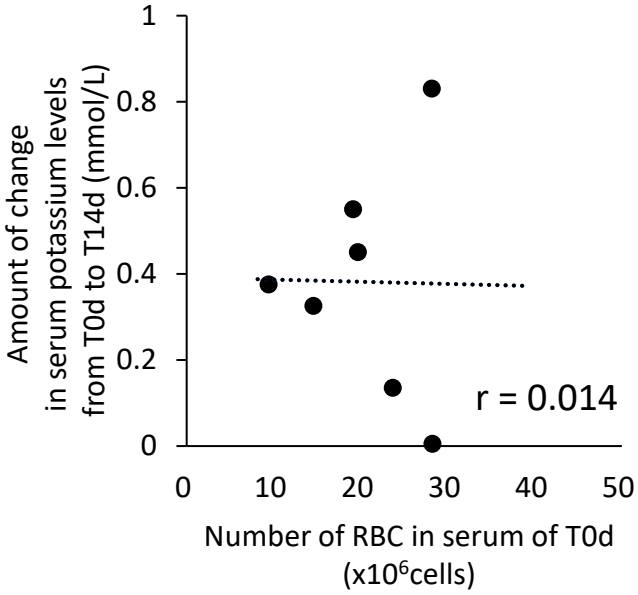

Rate of change from T0d (%)
25
20
15
10
5
0
-5
**
T0d T1d T3d T7d T14d
K
Na

Fig2

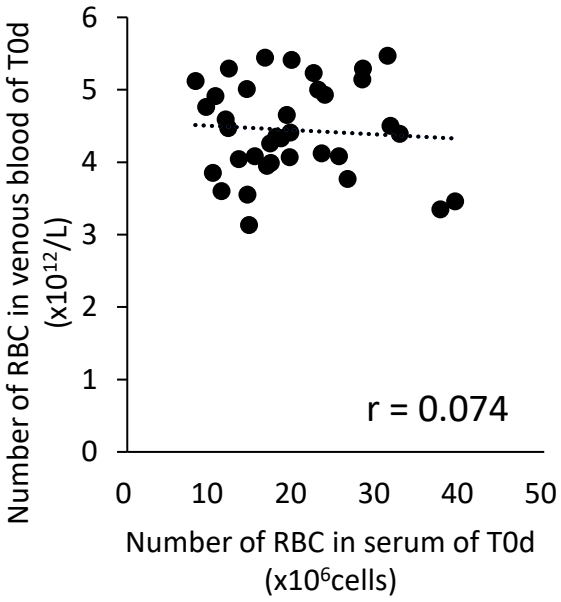
A



B


Amount of change in serum potassium levels from T0d to T14d (mmol/L)
1
0.8
0.6
0.4
0.2
0
r = 0.014
0
10
20
30
40
50
Number of RBC in serum of T0d (x10⁶cells)

C


Number of RBC in venous blood of T0d (x10¹²/L)
6
5
4
3
2
1
0
r = 0.074
0
10
20
30
40
50
Number of RBC in serum of T0d (x10⁶cells)

D

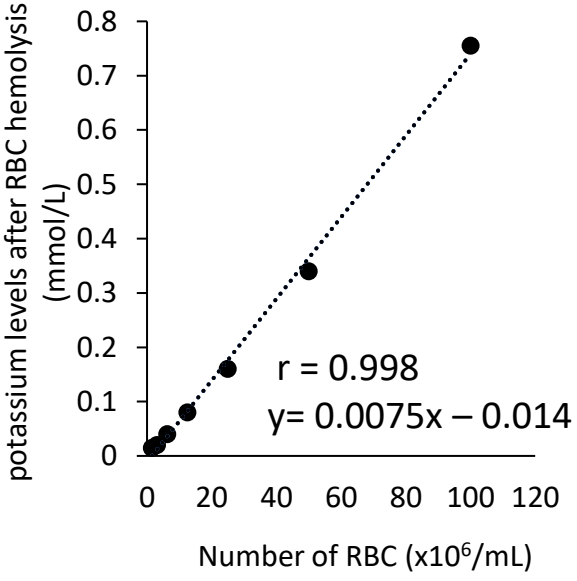

potassium levels after RBC hemolysis (mmol/L)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
r = 0.998
y= 0.0075x – 0.014
0
20
40
60
80
100
120
Number of RBC (x10⁶/mL)

Fig3

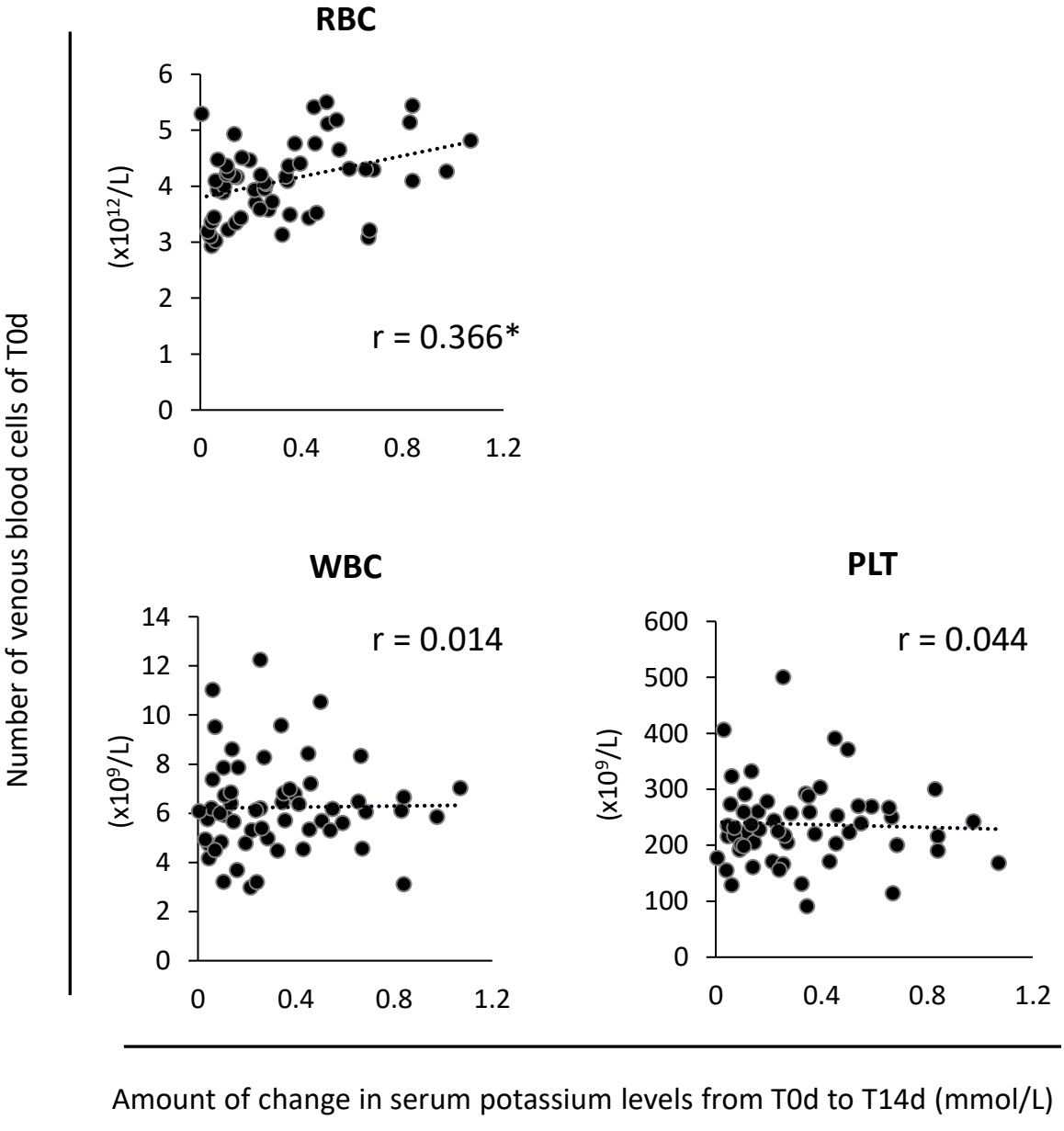
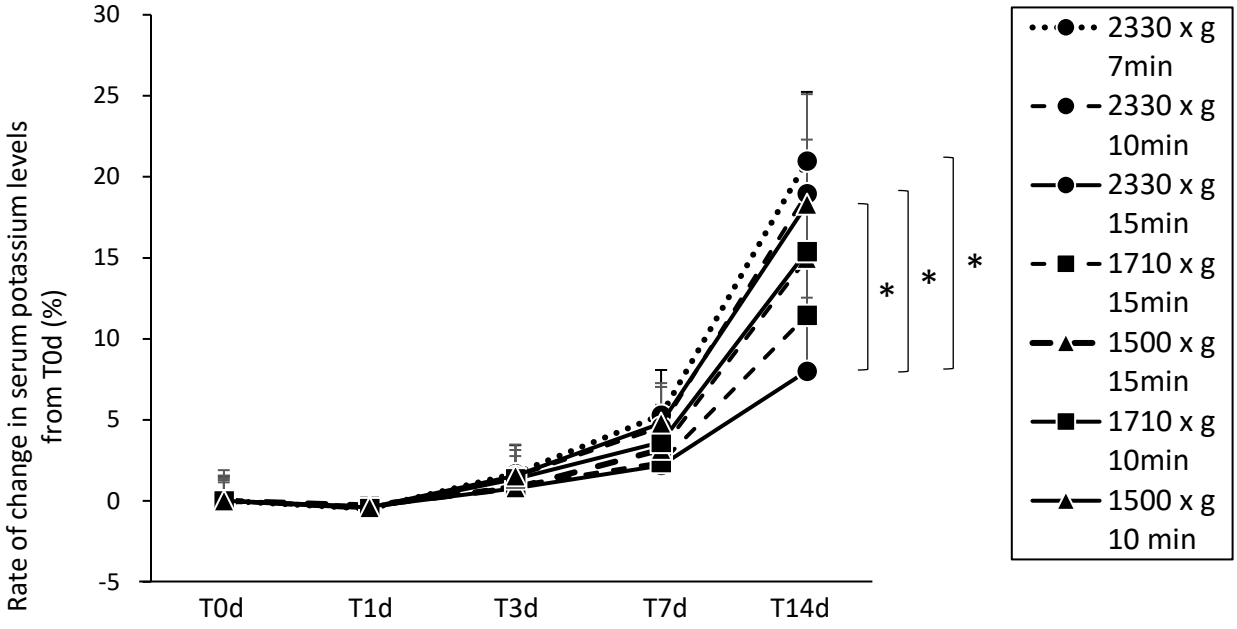
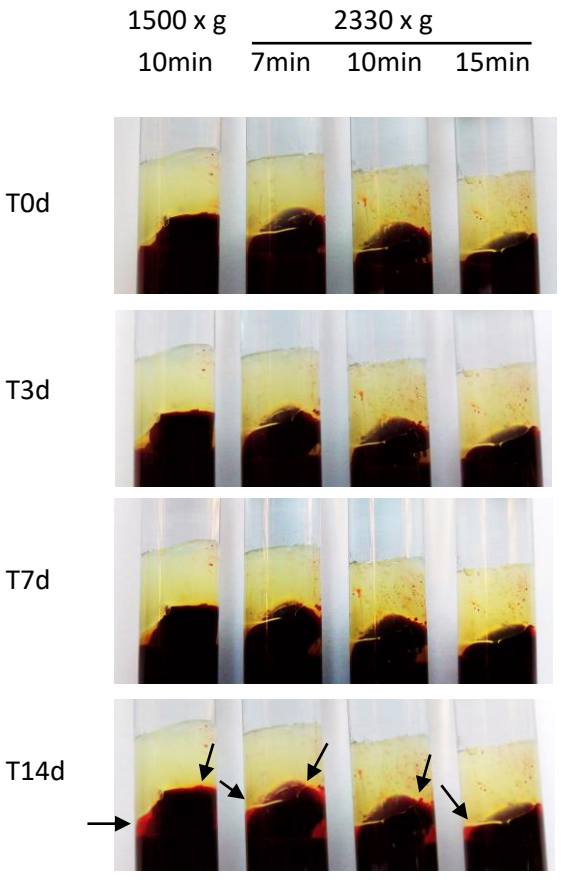

RBC
(x10^12/L)
r = 0.366*
WBC
(x10^9/L)
r = 0.014
PLT
(x10^9/L)
r = 0.044
Number of venous blood cells of T0d
Amount of change in serum potassium levels from T0d to T14d (mmol/L)

Fig4

A


Rate of change in serum potassium levels from T0d (%)
30
25
20
15
10
5
0
-5
T0d
T1d
T3d
T7d
T14d
*
*
*
2330 x g 7min
2330 x g 10min
2330 x g 15min
1710 x g 15min
1500 x g 15min
1710 x g 10min
1500 x g 10 min

B


1500 x g
10min
2330 x g
7min
10min
15min
T0d
T3d
T7d
T14d

C

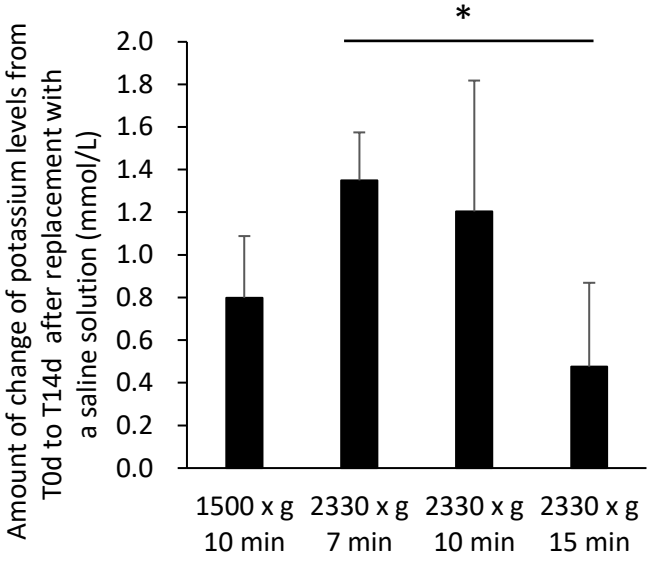
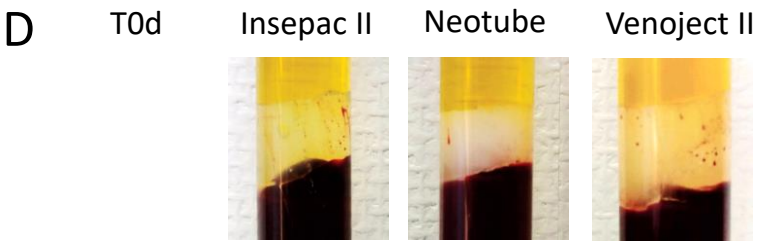
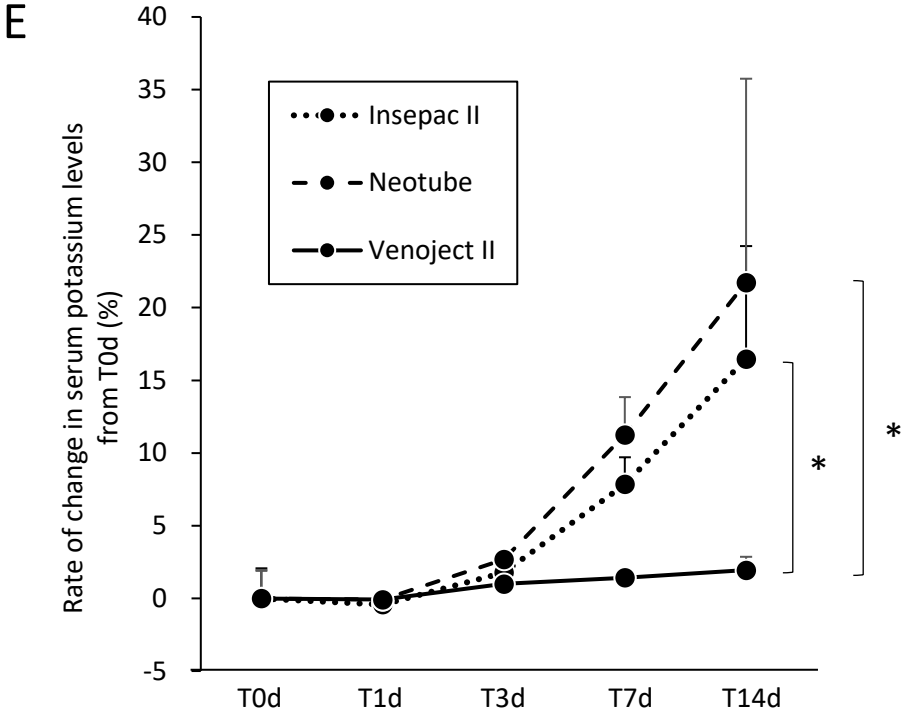

Amount of change of potassium levels from T0d to T14d after replacement with a saline solution (mmol/L)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
1500 x g 10 min
2330 x g 7 min
2330 x g 10 min
2330 x g 15 min

Fig4

D


T0d
Insepac II
Neotube
Venoject II

E


Rate of change in serum potassium levels from T0d (%)
40
35
30
25
20
15
10
5
0
-5
Insepac II
Neotube
Venoject II
T0d
T1d
T3d
T7d
T14d
*
*