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Involvement of red blood cell on calcium oxalate crystal growth and aggregation in vitro

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KEYWORDS
Red blood cell;
Calcium oxalate;
Crystal growth;
Crystal aggregation.

ABSTRACT
Cell membranes and their components may play an important role in stone formation. Hematuria is one of the most common manifestations in kidney disease. We therefore extensively investigate the involvement of intact red blood cell (iRBC) and red blood cell membrane fragments (fRBC) on CaOx crystal growth and aggregation. Intact RBC and fRBC were prepared from healthy blood samples. Calcium oxalate monohydrate (COM) and calcium oxalate dihydrate (COD) crystals were investigated for crystal growth and aggregation in the condition without or with iRBC or fRBC. Crystal growth and aggregation were analyzed from crystal area and the number of crystal aggregation, respectively, and also confirmed by using spectrophotometric oxalate depletion assay and calcium oxalate crystal aggregation-sedimentation assay, respectively. The results showed that only COM crystal with fRBC was significantly increased the crystal area and the number of crystal aggregation as compared to control conditions (p-value = 0.035 and p-value = 0.011, respectively) while COD crystals did not have any significant change to the crystal area or the number of crystal aggregates with both types of RBC. These data indicated that fRBC might promote the growth and aggregation of COM crystals. However, the molecular mechanism involvement of fRBC with COM crystals still needs to be studied.

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Introduction

Kidney stones consist of crystalline and non-crystalline (organic matrix) compositions. The organic matrix is a supporting structure of stones[1] and it is responsible for calcium stone[1, 2]. Which some macromolecules (lipids, proteins, glycosaminoglycans and carbohydrates) of stone matrix can play an important role in the promotion or inhibition of stone formation[3]. Urinary macromolecules may associate with calcium oxalate (CaOx) stone formation in two ways: 1) heterogeneous nucleation of CaOx crystals in low concentration of urinary salts leading to homogenous nucleation and 2) nucleated crystals further precipitate on the surface acting as an adhesive to bind more crystals leading to crystal aggregation and growth[4].

The previous evidence suggested that cell membranes and their lipids in urine play a role in calcium stone formation. The organic matrix of CaOx stone formers had a significant amount of acidic phospholipids compared to other types of stones[5]. Calcium-binding molecules on the inner surface of the red blood cell (RBC) membrane, particularly trans-membrane proteins may associate with crystal aggregation to red blood cell membrane fragments (fRBC)[5, 6]. Hematuria, RBC in urine, is one of the most common manifestations in urological and kidney disease and evidence of crenated red blood cells (RBC) in CaOx stone core matrix[7]. It is considered as effect of kidney stone disease. However, idiopathic hematuria with urinary metabolic abnormality (hypocitraturia, hypomagnesuria, hyperuricosuria, hypercalciuria and hyperoxaluria) showed increased promoters and decreased inhibitor concentration in the absence of nephrolithiasis[8]. These metabolic abnormalities were also risk factors for CaOx stone. A recent study showed that normal RBC does not promote calcium oxalate dihydrate (COD) crystal growth and aggregation[6]. However, the crenated RBC appeared to participate in the COD crystal growth[7] observed under a series of electron microscopic. Moreover, membrane lipid was found inside stone matrices of CaOx stones[5, 9].

We therefore extensively investigated the involvement of intact RBC (iRBC) and fRBC from healthy subjects on the growth and aggregation of calcium oxalate monohydrate (COM) and COD crystals in vitro.

Materials and methods

Selective criteria for healthy subjects

Healthy subjects were selected according to the results of nine parameters; hemoglobin (Hb) > 13.0 g/dl, hematocrit (HCT) > 39%, total RBC count 4.18 - 5.48 × 10^6 /µl, MCV > 90 fl, MCH > 27 pg, WBC count 4.6 - 10.6 × 10^3/µl, platelet count 150 - 400 × 10^3/ul, normal RBC morphology, and negative screening for hemoglobin E with dichlorophenol indophenol precipitation test.

Sample collection and preparation of iRBC and fRBC

This study was approved by the institutional ethic committee of Khon Kaen University (HE 621366). Blood samples from healthy subjects were collected into 2 ml tube containing ethylene diamine tetraacetic acid from Sirinagarind Hospital, Khon Kaen University. The blood samples were centrifuged at 2,500 rpm for 5 min. After that, 500 µl of RBCs from the bottom pallet was resuspended in 5 ml of an isotonic buffer containing 10 mM Tris-HCl (pH 7.4) and 150 mM NaCl. RBCs were washed for 5 times with isotonic buffer at 2,500 rpm. After RBC washing, RBC count was done with a hemocytometer and 10^5 cells/ml were used per assay. 10^5 cells/ml RBCs were treated with deionized water to lyse RBCs. When RBCs were lysed completely, the fRBC were separated by centrifugation at 10,000 g for 5 min. iRBC and fRBC were analyzed using a UriSed3 urine analyzer with UriSed3 Pro software (UriSed3 analyzer, Hungary) to check the purity of RBC, fragments, and RBC morphology.

Evaluations of iRBC and fRBC on COM and COD crystal growth and aggregation

To investigate the involvement of iRBC and fRBC on COM and COD crystal growth and aggregation, COM and COD crystals were generated as a previous study[6]. All assay conditions were performed in each well of a 24-well plate (Thermo Fisher Scientific, East Grinstead, UK). For control condition, COM crystals were prepared with

a final concentration of 5 mM $CaCl_2 \cdot 2H_2O$ and 1.0 mM $Na_2C_2O_4$ in a buffer containing 10 mM Tris-HCl and 90 mM NaCl (pH 7.4) while COD crystals were prepared with a final concentration of 6.27 mM $CaCl_2 \cdot 2H_2O$ and 1.6 mM $Na_2C_2O_4$ in a buffer containing 9.6 mM $C_6H_5Na_3O_7 \cdot 2H_2O$, 11.6 mM $MgSO_4 \cdot 7H_2O$, and 63.7 mM KCl (pH 6.5). For treatment condition, 105 cells/ml of iRBC or fRBC were added in each well. The solutions were incubated at 25°C for 1 h and then examined under the inverted light microscope (CKX41, Olympus; Tokyo, Japan) connected to a digital camera. Minimum 10 high power fields (HPF) were examined and pictures were captured. Crystal aggregates were defined as two or more crystals that adhered together. Secondly, crystal size was analyzed with Image J software (National Institutes of Health, USA). Minimum 100 individual COM crystals and 10 individual COD crystals were analyzed in each experiment. All experiments were done in triplicate for each sample.

Spectrophotometric oxalate-depletion assay of COM crystal growth

The spectrophotometric oxalate-depletion assay was carried out according to Nakagawa et al.[6, 10] to confirm the COM crystal growth with iRBC and fRBC. For control condition, COM crystal seeds (160 µg/ml) were added to 1-ml equilibrated solution containing 1 mM $CaCl_2 \cdot 2H_2O$, 1 mM $Na_2C_2O_4$, 10 mM Tris-HCl (pH 7.4) and 90 mM NaCl. For treatment conditions, iRBC or fRBC was added to the equilibrated solution to a final concentration of 10^5 cells/ml before adding crystal seeds. The depletion of free oxalate ions in a solution containing $CaCl_2$ and $Na_2C_2O_4$ due to the growth of seeded crystals were detected and monitored at 214 nm by UV-visible spectrometer (Eppendorf 6137000015 BioSpectrometer Fluorescence, Germany). The rate of free oxalate depletion was calculated using baseline value and the value after 60 min incubation with or without RBC (iRBC or fRBC) equivalent to 10^5 cells per assay. The relative percentage of oxalate depletion was calculated.

CaOx crystal aggregation-sedimentation assay of COM crystal aggregation

The effects of iRBC and fRBC on COM crystal aggregation were confirmed by CaOx crystal aggregation-sedimentation assay[11, 12]. For control condition, COM crystal seeds (100 µg/ml) was added to 1 ml solution containing 1 mM $CaCl_2 \cdot 2H_2O$, 0.1 mM $Na_2C_2O_4$, 10 mM Tris-HCl (pH 7.4) and 90 mM NaCl. The reduction of turbidity of the solution due to crystal aggregation was monitored by a UV-visible spectrophotometer at 620 nm for 1 h. For treatment conditions, iRBC or fRBC were added to the equilibrated solution to a final concentration of 10^5 cells/ml before adding crystal seeds. Crystal aggregation was evaluated by comparing the turbidity slope in the presence and the absence of iRBC or fRBC at the end of 1 h assay time. Besides, the morphology of the aggregates was observed under an inverted light microscope to reconfirm the crystal aggregation.

Statistical analysis

Statistics were calculated by SPSS program version 17.0 (IBM Corp, Armonk, NY, USA). Multiple comparisons were analyzed using variance with Turkey's post hoc test. Quantitative data were shown as the mean and standard error of the mean (mean ± SEM). A *p*-value of less than 0.05 was considered to be statistically significant.

Results

Preparation of iRBC and fRBC

To confirm the purity and homogeneity of iRBC and fRBC, the samples were analyzed by UriSed3 urine analyzer with UriSed3 Pro software before the experiment. The purity of iRBC isolated was greater than 99.8%. RBCs were completely lysed and fragmentation occurs approximately 99% of the RBC.

Involvement of iRBC and fRBC on COM crystal growth and aggregation

To investigate the effect of iRBC and fRBC on COM crystal growth, the COM crystals were generated in the absence and presence of iRBC or fRBC. Crystal areas in pixel unit (mean ± SEM) were compared to the control, iRBC and fRBC (4296.6 ± 50.0, 4834.2 ± 58.3, and 5850.4 ± 60.8), respectively. The fRBC significantly increased crystal size as compared to control condition (*p-value* = 0.035) (Table 1).

The number of crystal aggregates significantly increased with fRBC. Crystal aggregates in control, iRBC, and fRBC were (0.23 ± 0.08, 0.46 ± 0.12, and 1.27 ± 0.18), respectively. The fRBC significantly increased COM crystal aggregation as compared to control (*p-value* = 0.011) and compare to iRBC (*p-value* = 0.029) (Table 1).

Table 1 Involvement of iRBC and fRBC on COM crystal growth (size in pixel unit) and aggregation (number of crystal aggregates/HPF)

Experiment conditions	Control (C)	iRBC (I)	fRBC (F)	*p*-value
Crystal area (pixel unit)	4296.60 ± 50.00	4834.20 ± 58.30	5850.40 ± 60.80	a
Number of crystal aggregates/HPF	0.23 ± 0.08	0.46 ± 0.12	1.27 ± 0.18	b, c

Note: Quantitative data presented from 3 individual experiments as mean ± SEM. Each experiment was done in triplicate.
a; significant difference between F and C (*p-value* = 0.035).
b; significant difference between F and C *(p-value* = 0.011).
c; significant difference between F and I (*p-value* = 0.029).

Spectrophotometric oxalate depletion assay was performed to confirm the COM crystal growth. The assay was monitored for oxalate depletion with iRBC or fRBC for 60 min. At the end of 60 min, the amounts of oxalate depletion were 39.3 ± 1.4% of iRBC and 44.6 ± 1.4% fRBC.

To confirm the crystal aggregation with iRBC and fRBC, the CaOx crystal aggregation-sedimentation assay was performed (Figure 1). The fRBC significantly increased the crystal aggregation as compared to control and iRBC. At the end of 60 min, fRBC (78.0 ± 3.7%) decreased turbidity of reaction as compared to the control condition (59.3 ± 3.4%). The iRBC had no any significant effects on COM crystal aggregation.

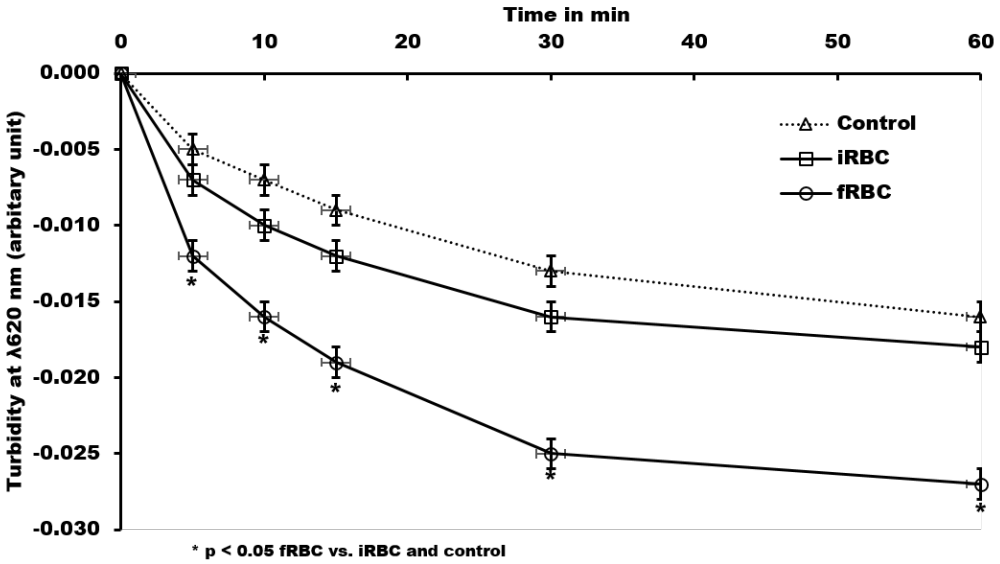

Figure 1 CaOx crystal aggregation-sedimentation assay.
Note: The reduction of turbidity from reactions was monitored at 620 nm for 60 min in control, iRBC and fRBC. Quantitative data presented as mean±SEM from 3 individual experiments. Each experiment was performed in triplicate.

The aggregated products were generated from CaOx crystal aggregation-sedimentation assay after 60 min (Figure 2). Aggregates induced by fRBC were much larger and firmer than those induced by control and iRBC.

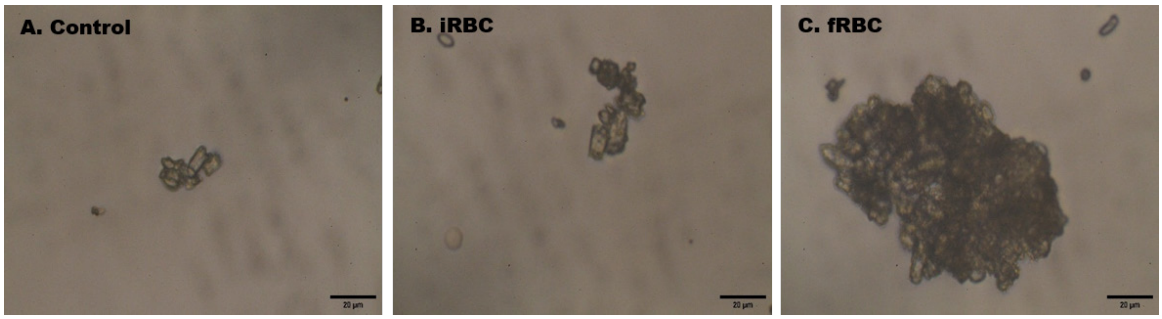

Figure 2 Aggregated product of CaOx crystals ((A) Control, (B) iRBC and (C) fRBC) by aggregation-sedimentation assay at 60 min (original magnification ×400).

Involvement of iRBC and fRBC on COD crystal growth and aggregation

To investigate the involvement of iRBC and fRBC on COD crystals, the generation of COD crystals was analyzed. Neither iRBC nor fRBC had a significant effect on COD crystal growth or crystal aggregation (Figure 3).

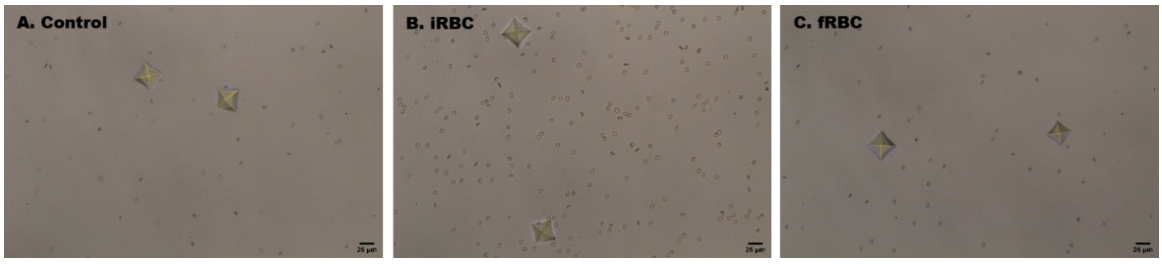

Figure 3 Effects of iRBC and fRBC on COD crystal growth and aggregation ((A) Control, (B) iRBC and (C) fRBC) under light microscope (original magnification ×400).

COD crystal growth represented as size area (pixel) of COD crystal were 4527.5 ± 206.1, 4645.2 ± 400.3 and 4356.5 ± 325.0 in control condition, iRBC and fRBC, respectively. The number of COD aggregates were 0.11 ± 0.003, 0.28 ± 0.006 and 0.36 ± 0.006 in control condition, iRBC and fRBC, respectively (Table 2).

Table 2 Involvement of iRBC and fRBC on COD crystal growth and aggregation

Experiment conditions	Control (C)	iRBC (I)	fRBC (F)	*p*-value
Crystal area (pixel unit)	4527.50 ± 206.10	4645.20 ± 400.30	4356.50 ± 325.00	d
Number of crystal aggregates/ HPF	0.11 ± 0.03	0.28 ± 0.06	0.36 ± 0.06	e

Note: Quantitative data presents as mean ± SEM from 3 individual experiments. Each experiment was performed in triplicate.
d; no significant difference between groups (F vs. C *p-value* = 0.904, F vs. I *p-value* = 0.858 and I vs. C *p-value* = 0.765).
e; no significant difference between groups (F vs. C *p-value* = 0.231, F vs. I *p-value* = 0.690 and I vs. C *p-value* = 0.397).

Discussion

Kidney stone formation is multifactorial such as genetic[3, 13-14] and environmental factors[3, 8, 15-17]. Some metabolic abnormalities (hypocitraturia, hypomagnesuria, hypercalciuria, hyperoxaluria, hyperuricosuria and potassium depletion in skeletal muscle)[3, 15-16] and reactive oxygen species[17] are risk factors for kidney stone formation. The three most common chemical compositions of stone were CaOx mixed with phosphate, pure CaOx and uric acid[18]. The organic matrix of CaOx stone formers had a significant amount of acidic phospholipids compared to other types of stones[5]. Hematuria is the most common manifestatios in kidney stone disease and evidence of crenated RBC in CaOx stone core matrix[7]. Therefore, in vitro experiments were performed to investigate the involvement of iRBC and fRBC on the growth and aggregation of CaOx crystals.
We carefully evaluated the morphology and purity of RBC that is suitable for the crystal analysis. Our result demonstrated that iRBC had no significantly effect on COM crystal growth or crystal aggregation. While fRBC promoted COM crystal growth approximately 36% and promoted COM crystal aggregate number approximately 2.5-fold as compare to control condition. This effect may be caused by some reasons. Firstly, calcium-binding molecules on the inner surface of the RBC membrane, particularly trans-membrane proteins associated with crystal aggregation to fRBC[5-6,9]. Secondly, cell membrane lipids may promote the stone formation. Some evidences found that the organic matrix of CaOx stone formers had a significant amount of acidic phospholipids compared to other types of stones examined[5]. The stone formers found an abnormal arachidonic acid content in RBC membrane[19].

Our results from healthy subjects were consistent with the previous study[6] which showed that iRBC had no significant effect on COM crystal, while fRBC showed 75% increase in size of the COM crystals and 2.5-fold increase in COM crystal aggregates number. The different percentages of crystal size may have effect from the source of blood samples. For the effect of iRBC on COD crystal growth and crystal aggregation, in this study was no statistically significantly effect similar to that of the Chutipongtanate and Thongboonkerd[6]. However, Kim s' study[7] showed that the crenated RBC appeared to participate in the COD crystal growth by observed under a series of electron microscopic in human urinary stones. For further studies should be analyzed the involvement of RBC of kidney stone formers on CaOx formation.

Conclusion

Our data indicated that fRBC significantly promotes CaOx crystal growth and aggregation. The fRBC may have involvement in CaOx crystal growth and aggregation. However, further studies are required to study the involvement of molecular mechanisms in fRBC with COM crystals.

Take home messages

Fragmented red blood cells significantly promoted calcium oxalate monohydrate crystal growth and aggregation in healthy subjects.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

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